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THE UNIVERSITY OF ALBERTA

MICROBIAL BIOSYNTHESIS AND METABOLISM OF PHARMACEUTICALLY IMPORTANT COMPOUNDS

by

EMIL C. DOMBROSKI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

IN

PHARMACEUTICAL SCIENCES

(DRUG METABOLISM)

FACULTY OF PHARMACY & PHARMACEUTICAL SCIENCES

EDMONTON, ALBERTA

SPRING 1988

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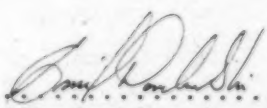

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Microbial Biosynthesis and Metabolism of Pharmaceutically Important Compounds submitted by Emil C. Dombroski in partial fulfillment of the requirements for the degree of Master of Science.

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(Supervisor)

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Date: March 29, 1988

To my wife, for her love, help,

encouragement and patience

ABSTRACT

Microbial fermentations containing filamentous fungi were monitored in order to observe natural product formation or the biotransformation of chosen foreign compounds (drugs) added to the fermentation broth. Investigations were conducted with a mind to applying these fermentations in the biosynthetic production of metabolites of interest or in the modeling of mammalian metabolism. Analytical methods used to detect, separate and identify compounds so produced incuded TLC, GC, HPLC, IR, NMR, and mass spectrometry.

Investigations were conducted into the biosynthesis of cyclosporine by five members of the genus Tolypocladium and the microbial metabolism of lidocaine, mexiletine, and fenoprofen by the filamentous fungi Cunninghamella echinulata UAMH 4144, Cunninghamella echinulata UAMH 4145, Aspergillus flavus UAMH 2679, Aspergillus niger UAMH 1240 and Rhizopus nigricans.

Tolypocladium inflatum Gams UAMH 2880 was selected as the most promising production organism for preparative biosynthesis of cyclosporine. Large-scale fermentation was carried out successfully utilizing all-glass airlift reactors as fermentation vessels. Structural identity of the purified metabolite was confirmed by comparison of its HPLC retention time and ^{1}H-NMR spectra with authentic standard.

Emphasis was placed upon developing efficient methods for culturing the production organism, enhancing the

production of the desired metabolite through manipulation of fermentation conditions and optimizing solvent extraction and separation by TLC and HPLC.

In studies on microbial metabolism, C. echinulata UAMH 4145, A. flavus UAMH 2679 and R. nigricans were observed to carry out such transformations as N-dealkylation of lidocaine, hydroxymethylation, ring hydroxylation and N-acetylation of mexiletine, and the hydroxylation of fenoprofen. With the exception of N-acetylation, each of these biotransformations have been reported in mammals. Preparative scale synthesis of 4'-hydroxyfenoprofen was confirmed by the interpretation of IR, ^{1}H-NMR and High Resolution EIMS.

The apparent metabolic inversion of racemic fenoprofen by C. echinulata was observed using a stereospecific HPLC method of analysis. This type of inversion has also been reported to occur in man.

In a study involving the metabolism of fenoprofen in man, a new metabolite, 4'-methoxyfenoprofen is tentatively identified.

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LIST OF ABBREVIATIONS

CIMS	chemical ionization mass spectrometry
Cy	cyclosporin
EIMS	electron impact mass spectrometry
GC	gas chromatograph(y)
GC/MS	gas chromatography / mass spectrometry
GX	glycinexylidide
HPLC	high pressure liquid chromatograph(y)
IR	infrared spectroscopy
LIDO	lidocaine
MEGX	monoethylglycinexylidide
MS	mass spectrometry
NMR	nuclear magnetic resonance spectroscopy
NSAID(s)	non-steroidal anti-inflammatory drug(s)
PFB(C)	pentafluorobenzoyl (chloride)
TLC	thin layer chromatograph(y)
XYL	xylidine

1.0.0.0 GENERAL INTRODUCTION

The project areas in this thesis all involve the microbial synthesis and metabolism of pharmaceutically significant biologically active compounds. In general, we wish to advance the idea that microbial fermentation is a powerful tool available to those conducting pharmaceutical research. The areas in which these fermentations can be used to advantage are often not well recognised although recent advances in this area are contributing to a rapidly expanding body of knowledge which makes the ability to exploit microbial systems an increasingly valuable alternative for industrial and academic research groups concerned with the production and metabolism of certain pharmaceuticals.

Our first objective was the preparative biosynthesis of cyclosporine (a secondary metabolite of Tolypocladium inflatum Gams, a filamentous fungal mold), which is being used clinically for its immunosupressive activity. We then wished to carry out activity and toxicity studies on this and other related metabolites which could possibly be produced in the same manner using directed biosynthetic techniques.

Our second objective was to study the metabolism by Cunninghamella echinulata (another mold) of mexiletine, lidocaine (antiarrhythmic drugs), and fenoprofen (a non-steroidal anti-inflammmatory compound). We desired in this instance to compare our metabolic findings with those which have been reported previously in clinical and animal studies

in the attempt to discover whether or not a microbial system might serve as a source for metabolites that are difficult to synthesize or possibly an alternative model for the study of the mammalian metabolism of these compounds.

The following sections in this introduction are intended to present the rationale for using each of these microbial systems and a brief background for each of the drugs of interest.

1.1.0.0 **Microbial fermentations**

Man has utilised microorganisms in fermentations for thousands of years in the production of alcohol (beer and wine) and in the leavening of bread. More recently, the discovery of penicillin in the 1930's and 1940's led to a proliferation of economically viable fermentation processes and products (the majority of these being secondary metabolites) in the pharmaceutical industry. The boom in microbiological studies which followed was initially focussed on the discovery of new active substances and metabolites. As data was collected and compiled it was observed that various microorganisms were able to carry out a number of different transformation reactions. Moreover, these transformations were frequently regioselective/specific and stereoselective/specific (1,2). This suggested a whole new area of application for microbial fermentations (aside from synthesis of secondary metabolites), namely the possibility of using a fermentation as a step in the overall chemical synthesis of an organic product. Thus a xenobiotic would be added to the fermentation broth as substrate and the metabolised or transformed product would later be extracted, purified and further modified if necessary. Therefore, in its most general sense, any biological system can be considered to perform the chemical transformation of organic compounds with the aid of enzymes as catalysts (3) (enzyme mediated transformations will hereafter be refered to as

biotransformations). We were particularly interested in those enzymes of microbial origin.

Another observation which has begun to attract attention is the fact that many of the biotransformations found in microorganisms also occur in higher animals including mammals and man (4). Furthermore it has been shown that the cytochrome P_{450} mono-oxygenase enzymes in microorganisms are similar to those found in mammalian liver (5,6,7,8) and in 1974 Smith and Rozzaza first proposed the concept of utilizing microbial systems to study mammalian metabolism (9).

1.1.1.0 **Microbial systems**

Before considering the various advantages and disadvantages of using a microbial system it should be emphasised that we have approached the use of such a system from two different perspectives.

In the first case, we used a microbial fermentation for the preparative synthesis of a natural product (cyclosporine). In this instance the microbial system could be simply considered as a black box, or the organism could itself be considered to be analagous to a catalyst in a synthetic organic reaction. It is possible to cause the production or, even better, the overproduction of the desired metabolite(s) and minimizing unwanted ones through the manipulation of the following parameters (6): a) selection of

the species and strain of the organism to be used - these can differ significantly in morphology, growth rate, metabolic regulation and genetic stability; b) fermentor design - which affects proper regulation of oxygen transfer, mass transfer, temperature, pH and physical stress; c) media composition - where proper carbon, nitrogen and phosphorous sources, along with trace elements, inducers, inhibitors, substrates or precursors can all influence the growth and metabolism of the organism. Once the desired product is formed, it must then be recovered from the broth and purified in some manner ensuring against its possible degradation in the process.

The second application involves considering the microbial system as a model organism which, when challenged with a xenobiotic, will characteristically metabolize that xenobiotic in a manner that may be comparable to the metabolism of higher animals, such as man, by virtue of the presence of several closely related, if not identical, enzyme systems (6,8). It should be noted that this model utilizes a single-celled, eukaryotic organism and therefore does not take into consideration such pharmacokinetic aspects of metabolism as absorption, distribution or excretion as are often critical in multicellular organism studies. Instead, it is concerned specifically with the biotransformation of the xenobiotic. Its greater simplicity allows a more direct study of the individual reactions taking place although this is not quite analogous to the simplest case of a purified enzyme system. These models appear especially useful in producing

preparative quantities of metabolites for complete structure elucidation and biological testing which in turn can provide useful information on mechanism of action, activity and/or toxicity studies (9,10).

1.1.2.0 **Advantages of a microbial system**

Certain fundamental characteristics are shared by different microbial processes which make them readily applicable to the studies we have carried out. Most important of these is perhaps the small size of the microbial cell. Its correspondingly high surface-to-volume ratio facilitates the rapid transfer of nutrients and substrates thereby allowing for an extremely rapid metabolic rate. Evidence of this can be seen in the fact that some microorganisms are able to reproduce themselves in as little as fifteen to twenty minutes (11). In addition to this, the ruggedness of these organisms is extraordinary. They may be found flourishing in almost all known environments and their ability to withstand freeze-drying, storage for indefinite periods of time (years) and subsquently produce a viable culture using simple rehydration techniques have led to the development of extremely valuable culture collections. This makes a staggering assortment of microorganisms accessable to researchers. These cultures are then easily stored on location and regenerated in sufficient quantities for conducting individual experiments or large study programs and

are amenable to routine handling with a confident degree of stability and reproducibility.

Other advantages of microbial systems as they apply to specific project areas in this thesis are described in Sections 3.1.1.0 and 4.1.2.0.

1.2.0.0 Objectives

1.2.1.0 Objectives for the biosynthesis of cyclosporine

The recent increase in success of clinical allografts has, in part, been attributed to the selective immunosuppression exhibited by CyA. Its relatively low incidence of toxicity has contributed to its being proposed as a forerunner of a new third generation of agents available for organ transplantation (12,13,14,15,16).

The first proposal in this thesis involved conducting an investigation into the possibility of setting up a production system and developing appropriate techniques that would enable the preparative scale biosynthesis of CyA and related metabolites (which have been reported as being natural products of some members of the genus Cylindrocarpon and Tolypocladium) (17). The amount of these compounds to be produced was to be in the order of milligrams or larger so as to provide enough material to carry out a variety of structural and activity studies.

1.2.2.0 Objectives for the microbial metabloism of selected drugs

Each of these drugs (lidocaine, mexiletine, and fenoprofen) are good candidates for microbial oxidative metabolism by C. echinulata (e.g., ring/aryl hydroxylation as well as various sites for possible oxidation and dealkylation). Comparison of the microbial metabolites of these drugs with known human and mammalian metabolites will help to highlight parallel biotransformations taking place. The use of this organism may then be considered as a synthetic step in the production of metabolites of interest which, via routine fermentation scale-up techniques, can provide enough material to enable structural elucidation by IR, NMR, and MS analysis, and activity testing in animal models as well.

We did not have access to an authentic sample of 4'-hydroxyfenoprofen, a suspected metabolite of fenoprofen and there was no available literature on the structural analysis of this human metabolite, reported previously by others. Therefore, a concurrent experiment was run involving the the administration of fenoprofen to human volunteers, with the subsequent collection, extraction and analysis of urinary metabolites in order to afford a direct comparison with any microbial metabolites isolated.

2.0.0.0 MATERIALS AND METHODS

2.1.0.0 General materials

2.1.1.0 Chemicals and reagents

Reagent grade solvents were obtained from a variety of local suppliers: Fisher Scientific Co., Edmonton, Canada; Caledon Laboratories Ltd., Georgetown, Canada; BDH Chemicals, Toronto, Canada.

HPLC grade solvents (Fisher Scientific Co., Edmonton, Canada).

Sodium sulfate, sodium chloride, sodium bicarbonate, potassium bicarbonate, potassium phosphate dibasic, sodium nitrate, magnesium sulfate, dextrose, potassium chloride (Fisher Scientific Co., Edmonton, Canada).

D_2O, $CDCl_3$, DMSO-d_6 (Aldrich Chemical Co., Milwaukee, WIS.).

Acetic anhydride, pentafluorobenzoyl chloride (Pierce Chemical Co., Rockford, IL).

The following compounds were received as gifts from their manufacturers:

Cyclosporine (Sandoz Ltd., Basle Switzerland).

Lidocaine HCl, monoethylglycinexylidide HCl, glycinexylidide HCl (Astra Pharmaceuticals Ltd., Mississauga, Canada).

Mexiletine HCl (Boehringer Ingelheim KG, Berlington, Canada).

Fenoprofen calcium (Eli Lilly, Toronto, Canada).

2.1.2.0 **Culture media**

Czapek Dox Broth, Sabouraud Dextrose Broth, Nutrient Broth, Potato Dextrose Broth, Nutrient Agar, Seed Agar (Difco Laboratories, Detroit, MI).

Tripticase Soy Broth (BBL Microbiology Systems, Becton Dickinson and Co., Cockeyesville MD).

Oatmeal cereal (H.J. Heinz Company of Canada, Leamington, Canada).

Quick Oats (Robin Hood Multifoods Inc., Willowdale, Canada).

2.1.3.0 **Miscellaneous equipment**

Glassware

Kimax 13 x 100 mm and 17 x 125 mm reusable borosilicate screw cap test tubes with teflon-lined plastic screw caps, 125 ml, 250 ml, 500 ml, 1 L, and 2 L metal-capped culture flasks (Fisher Scientific Co., Edmonton, Canada).

170 ml, 1.25 L, 4.5 L airlift bioreactors (manufactured by Technical Services, University of Alberta, Edmonton, Canada) (Fig. 1,2, pages 22,23).

Milli-Q Reagent Water System (Millipore, Bedford, MA).

Petri dishes: 15 mm plastic (Fisher Scientific Co., Edmonton, Canada).

Filters: 0.22 micron Durapore, Millex-FG 0.2 mm sterile filter units, Twin-90 0.22 um filter units (Millipore, Bedford, MA).

Vacuum filtration unit: Waters Solvent Clarification Kit (Waters, Mississauga, Canada).

TLC plates: plastic-backed, precoated silica gel 60 0.2mm thick (F_{254}), HPTLC silica gel 60 (F_{254}) 0.25mm thick precoated with preconcentrating zone (E. Merck, Darmstadt, West Germany).

Autoclave: Amsco Steam Autoclave (Amsco, Erie, PA).

Dynac Benchtop Centrifuge (Becton Dickinson and Co., Parsippany, NJ).

Microfuge B (Beckman Instruments Inc., Palo Alta, CA)

Savant Speedvac Concentrator (Savant Industries Inc., Hicksville, NY).

1.5 ml microfuge tubes (Fisher Scientific Co., Edmonton, Canada).

Multi-testtube vortex mixer: (Ika-Werk Ika-Vibrax-VXR Janke and Kunkel GmbH u.CoKg Staufen, FRG c/o Terochem Laboratories, Edmonton, Canada).

Vortex mixer: Vortex Genie Model K-550-G (Scientific Industries Inc., Springfield, MASS).

Flash-evaporator (Roto-vap) (Janke & Kunkel IKA WERK c/o Terochem Laboratories, Edmonton, Canada).

pH meter: Model 140 (Fisher Scientific Co., Edmonton, Canada).

Combination micro electrode with Ag/AgCl reference (Fisher Scientific Co., Edmonton, Canada).

Controlled environment incubator-shaker: Model G-25 (New Brunswick Scientific Co. Inc., Edison, NJ).

Biogard Model B60-112 Class II Containment Cabinet (The Baker Co., Sanford, ME).

Incubators: Dry type bacteriological incubator (Blue M Electric Company, Blue Island ILL).

Flow meters: Matheson Model 601 and 602 flow meters (Matheson Gas Products, Edmonton, Canada).

Compressed gasses (Union Carbide Canada, Ltd., Edmonton, Canada).

Statzyme glucose test kit (Worthington Diagnostic Systems Inc. Freehold, NJ).

2.1.4.0 **HPLC equipment**

The configurations of the HPLC systems used were as follows:

Cyclosporine analysis

U6K injector, model M-45 isocratic pump, Lambola-Max Model 481 variable wavelength (U.V.- Vis.) L C spectrophotometer, 5 cm C_{18} reverse-phase-packed guard column and oven (Waters, Milford, MASS).

3390A Integrator-Recorder, RP-8 (10 um), 20 cm C_8 reverse phase column (Hewlett Packard, Mississauga, Canada).

Fenoprofen analysis

The HPLC consisted of a 590 pump, a 490 programmable multi-wavelength UV detector, a 710B Wisp autosampler, a 4.6 mm x 10 cm analytical column containing 5 micron octadecylsilane packing material (Partasil 5 ODS-3; Whatman, Clifton, NJ) and a 2 cm Uptight Precolumn (Upchurch Scientific, Rexdale, Canada) packed with 37-53 micron reversed-phase material. The recorder was a Hewlett Packard model 3390A integrator (Hewlett Packard, Mississauga, Canada).

2.1.5.0 **GC equipment**

Durabond capillary columns (J & W Scientific, Folsom, CA) were used.

The GC system consisted of a Model 5700A equipped with a flame ionization detector, a capillary column controller model 18740B and a model 3390A integrator-reporter (Hewlett Packard, Mississauga, Canada). Constant operating conditions were: carrier gas (He) 60 ml/min; H_2 40 ml/min; air 240 ml/min; injection port and detector temperatures were 250°C.

2.1.6.0 **Spectrometers**

EI mass spectra of compounds separated on a Model 5710A GC were recorded on a model 5980A spectrometer (Hewlett Packard, Mississauga, Canada).

Electron impact, chemical ionization, and high resolution mass spectra were also obtained on an AEI MS-50 spectrometer (Department of Chemistry, University of Alberta, Edmonton, Canada).

NMR spectra were recorded on a Bruker AM-300 (300 MHz) spectrometer (Bruker Spectrospin Canada, Milton, Canada).

IR spectra were determined on a Nicolet 5 DX spectrometer (Nicolet Instrument Canada, Mississauga, Canada).

2.1.7.0 **Source of biological materials**

The microorganisms used in these studies were obtained from the University of Alberta Mold Herbarium (Devonian Botanical Gardens, Devon, Canada).

Human biological samples for metabolite analysis were obtained from volunteers under the supervision of Dr. F. Jamali, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Canada.

2.2.0.0 General methods

2.2.1.0 Solvent preparation

HPLC solvents were used as obtained by the manufacturer. Reagent-grade solvents were glass-distilled prior to use. HPLC mobile phases were degassed *via* vacuum filtration using Millex-Dualex 0.45 um filters.

Reagent grade water was available on demand by glass-distilling in-house distilled water and passing it through a Milli-Q Reagent Water System equipped with a carbon filter cartridge, two ion exchange cartridges, an Organ-X cartridge, and a 0.22 um Twin-90 filter unit. This water filtration system was capable of delivering water the purity of which was measured by monitoring its resistivity (approximately 15 ohms)

2.2.2.0. Cleaning of glassware

All glassware was washed with a commercial glassware detergent, rinsed thoroughly with tap water, reagent-grade water, 95% ethanol, and allowed to dry at room temperature.

Any glassware involved in HPLC or GC analysis was washed in detergent, rinsed with tap water, reagent-grade water, methanol, dichloromethane, allowed to dry at room temperature, and heated in a muffle furnace to 500°C for at least one hour.

2.2.3.0 **Storage of standards and samples**

All standard solutions were stored at 4°C. Authentic standards and samples which were not immediately extracted, derivatized, or otherwise handled, were kept in a -17°C freezer.

2.2.4.0 **General media preparation**

Dehydrated media was reconstituted with an appropriate volume of distilled water as specified by the manufacturer. Broths were then dispensed into culture flasks or tubes as required, autoclaved for 15 minutes at 121°C and 20 psi and cooled to room temperature prior to the aseptic addition of microorganism and/or filter-sterilized substrate.

In media recipes calling for dextrose, dextrose solutions were prepared and autoclaved separately and added aseptically to the media after autoclaving. When autoclaved in the presence of phosphate, glucose produces some discoloration and toxicity (11).

Agar media for slants was autoclaved in batches and subsequently dispensed aseptically into sterile culture bottles or plates before cooling.

When preparing cereal agar for maintenance slants, autoclaving was repeated a second time in order to ensure

that no viable spores, often present in cereal grains, could survive.

All aseptic transfers were carried out in a Biogard Hood with flame sterilization whenever possible.

2.2.5.0 **Maintenance cultures**

Cultures of fungal organisms were inoculated onto cereal agar and/or potato dextrose agar slants made up of 10 ml of media in 25 ml culture bottles. This was done using a sterile loop to transfer mycelia and spores. These cultures were then incubated at 27°C until heavy growth and sporulation was evident whereupon they were kept refrigerated at 4°C. Periodic transfer of these cultures was carried out every 4-6 months in order to maintain the vigor and transformation characteristics of the organism. These maintenance cultures then served as a source for inoculation of working cultures.

Plating out samples of maintenance and working cultures onto nutrient agar was performed periodically and after 24-48 hours of incubation plates were examined by the naked eye and microscopically in order to ensure purity of the culture.

2.2.6.0 **Working cultures**

Working cultures (submerged, liquid) were prepared in a two-stage process. Phase I cultures were designed to produce a large biomass of rapidly growing cells for phase II

cultures which were then used in the actual fermentation experiments.

Phase I cultures were produced by aseptically adding 2 ml of sterile distilled water to a maintenance culture bottle which was then sealed and shaken vigorously to dislodge spores. The resulting spore suspension was then recovered and 1 ml was transferred to a 125 ml metal-capped culture flask containing 25 ml of the appropriate sterile liquid medium (Table 1). These phase I cultures were then incubated for 2-3 days in an environmental rotary shaker incubator, in the dark at 27°C until a large biomass was obtained. This large biomass was an arbitrary judgement as to the presence of sufficient mycelial cells that the fermentation culture would have a significant metabolic capability. Upon sporulation, the freely suspended homogeneous mycelial cells form small spherical pellets which are desirable for their large surface area and good mass transfer characteristics.

Phase II cultures were prepared by aseptically homogenizing phase I cultures into a fine mycelial suspension, portions of which were added to fresh media (as a 5% inoculum), held in culture flasks for screening experiments or returned to the shaker incubator for 24 hours to promote good pellet formation before addition to airlift reactors for preparative scale production or metabolism studies (Fig. 1,2).

All screening experiments were carried out in duplicate with media blanks. Organism controls were prepared by

incubating the organism in the absence of substrate, and substrate controls were prepared by incubating substrate in the absence of organism.

Airlift reactors were provided with oxygen at the following flow rates (ml/min): the 170 ml reactor was set at 100, the 1.25 L reactor was set at 275 and the 4.5 L reactor was set at 600.

Table 1: Media for working cultures of screen organisms. Incubations were carried out at 27°C.

Organism	UAMH#	Maintenance Media	Working Media
Tolypocladium inflatum	2880	Cereal agar	Basic B broth
Tolypocladium inflatum	4002	Cereal agar	Basic B broth
Tolypocladium inflatum	2472	Cereal agar	Basic B broth
Tolypocladium cylindrosporum	4561	Cereal agar	Basic B broth
Tolypocladium geodes	4591	Cereal agar	Basic B broth
Cunninghamella echinulata	4144	Cereal agar	Czapek Dox broth
Cunninghamella ecinulata	4145	Cereal agar	Czapek Dox broth
Aspergillus niger	1240	Cereal agar	Czapek Dox broth
Aspergillus flavus	2679	Cereal agar	Czapek Dox broth
Rhizopus nigricans		Cereal agar	Trypticase Soy broth

FIGURE 1: Airlift bioreactor with external loop (170/1,250 ml)

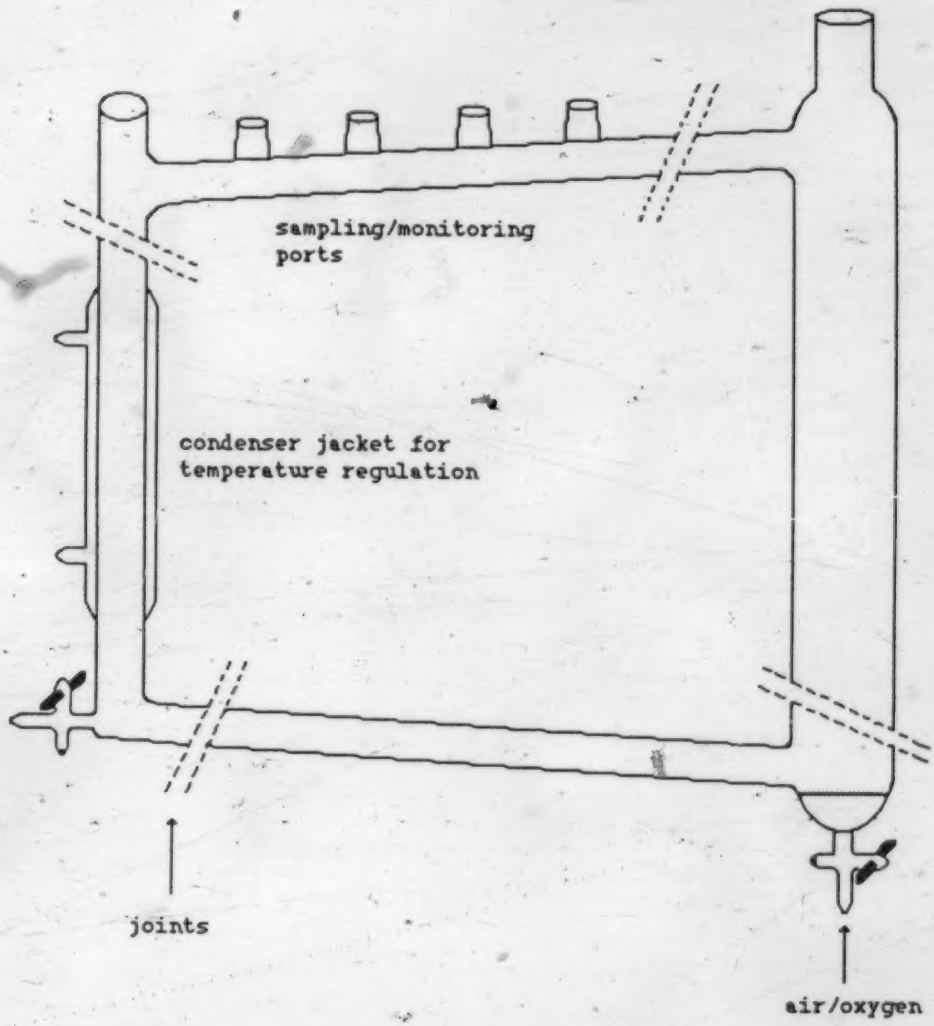


FIGURE 2: 4.5 L., four-piece airlift bioreactor

3.0.0.0 MICROBIAL BIOSYNTHESIS OF A NATURAL PRODUCT: CYCLOSPORINE

3.1.0.0 Introduction to the biosynthesis of cyclosporine

3.1.1.0 Chemical *vs* microbial synthesis

Chemical synthesis of an organic compound may seem to be an obvious method of production and in many cases would indeed prove satisfactory provided that the desired molecule has a relatively simple structure. Cost in money, time, materials and man-hours (manipulation) can rise dramatically as technical difficulties are encountered with increasingly complex structures. For example, problems often occur when dealing with large polyfunctional molecules where regiospecificity can be a major constraint in carrying out a desired chemical transformation. Often unwanted byproducts and residual starting material can structurally resemble the desired product to such an extent that separation and purification can be extremely challenging.

The harshness of conditions required to bring about a given chemical transformation must also be considered. The use of organic solvents, high temperatures, extremes of pH and pressure may be severely limiting if the molecule to be transformed contains labile functional groups, bringing into

play the possible necessity of elaborate protecting and deprotecting schemes which would detract further from efficiency and yield in the overall process.

Stereospecificity can be even more difficult to achieve. It is important to realize that a product is often of interest due to its biological activity and in a biological system this compound likely sees a role within some enzyme-mediated reaction. Enzymes are essentially chiral reagents and as such their substrates and products are not usually racemates but specific enantiomers (18). However, stereospecificity is a feature infrequently found in ordinary chemical catalysts, making the chemical synthesis of pure enantiomers an extremely difficult process (19).

A microbial system can offer a number of solutions or ways around some of the obstacles found in chemical synthesis, the provision here being that a suitable organism can be found. As stated earlier (in Section 1.1.2.0), the possibilities of this are extremely good with well catalogued culture collections and a rapidly growing literature base from which to draw. An increasing number of organisms have their metabolism well enough characterized that specific recommendations can be made in confidence with regard to desired biotransformations and biosyntheses (20). Even where purely chemical synthesis is possible, the incorporation of certain microbial biotransformations can prove cost-effective by reducing the stepwise length of the synthesis and/or by allowing the use of cheaper starting materials (21).

While chemical syntheses run into problems with regioselectivity/specificity or stereoselectivity/specificity, enzyme catalyzed reactions exhibit specificity in the type of reaction catalyzed, the structure of the substrate, the rate of catalysis and, in many cases, in their ability to discriminate between enantiomers (19). It is possible to exploit these characteristics of enzymes to achieve as great, if not greater, yields than in purely chemical syntheses, and in some cases nearly 100% conversion of substrate into the desired product can be attained (22,23). Consider further that with an enzyme catalysed reaction, bioconversions take place under extremely mild conditions of biological temperature, pH, and atmospheric pressure, and with water as the solvent (this may pose a problem if the substrate is hydrophobic). In general these are extremely advantageous conditions for retaining the integrity of labile groups or molecules.

In order to actually begin production of the desired metabolite, a facile scale-up of the volume of the reactor vessel from milliliters to liters may be all that is necessary. It is also possible, through genetic and environmental manipulations, to eliminate or bypass normal metabolic regulatory mechanisms of the fermentation organism in order to increase the yield of product. This is referred to as overproduction of the metabolite by the organism. Directed biosynthesis (the addition or deletion of trace nutrients, enzyme inducers and inhibitors) can be utilized to suppress normal regulatory mechanisms and induce

overproduction of the product. Mutasynthesis, involving the alteration of the organism's genetic material through induced mutation and screening the resulting mutants for less regulated strains, again results in overproduction and helps to optimize product yield (24-26).

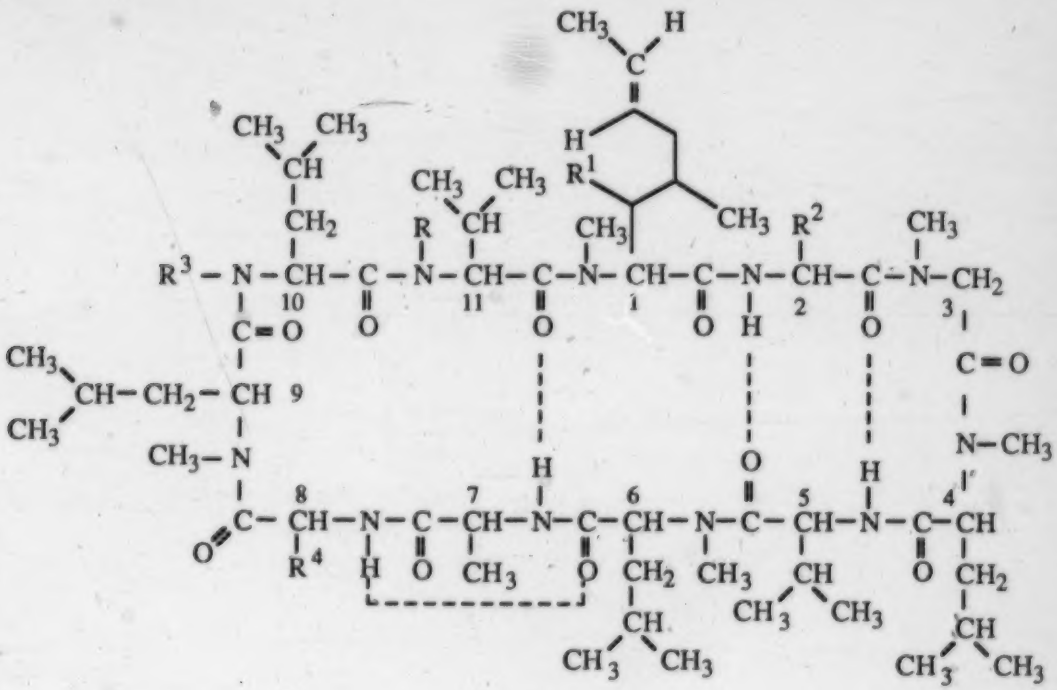
Economic large-scale production on the level of pilot plants or full-scale commercial facilities generally requires the complex manipulation of the fermentation process involving such parameters as the design and geometry of the fermentation vessel, heat and mass transfer, air flow, fluid flow, viscosity, shearing forces and power input. Rational and reliable methods and strategies for scale-up to these processing levels have not always been obvious or well documented in the past but current studies promise to satisfy the technical and economic demands of the modern biotechnology industry (27).

In recognition of these persuasive arguments, especially in cases where no other alternative seems economically or mechanistically feasible, microbial biosynthesis has become a valuable tool in the production of many complex, difficult to synthesize, biologically active compounds (3).

3.1.2.0 **History of cyclosporine** -

Cyclosporine (Sandimmune, Cyclosporin A, CyA) (Fig. 3) is one of a number of naturally occurring metabolites isolated from fermentations of the filamenous fungus

Tolypocladium inflatum Gams (previously described as Trichoderma polysporum (17,28) and Tolypocladium niveum (26,29,30)) which was reported to exhibit antifungal, immunosuppressive, and anti-inflammatory activity. Ten of these metabolites were identified as cyclosporins (designated Cyclosporin A through J) and consist of cyclic, neutral (highly hydrophobic) undecapeptides which possess a unique amino acid residue at position 1, and which differ mainly in the composition of the amino acid residue located at position 2. CyA was originally discovered in 1970 when in vitro screening tests identified its narrow spectrum of antifungal activity. This property was found to be poorly retained in in vivo studies but further screening was carried out nevertheless, on the strength of its relatively low degree of toxicity. The results of these tests revealed its powerful immunosuppressive properties (30,31). CyC, CyD, CyE, CyG, and CyJ have also been reported to possess immunosuppressive properties (32-43).



Cy	R	R1	R2	R3	R4
A	CH_3	OH	CH_2CH_3	CH_3	CH_3
B	CH_3	OH	CH_3	CH_3	CH_3
C	CH_3	OH	$CHOHCH_3$	CH_3	CH_3
D	CH_3	OH	$CH(CH_3)_2$	CH_3	CH_3
E	H	OH	CH_2CH_3	CH_3	CH_3
F	CH_3	H	CH_2CH_3	CH_3	CH_3
G	CH_3	OH	$CH_2CH_2CH_3$	CH_3	CH_3
H	CH_3	OH	CH_2CH_3	CH_3	CH_3
I	CH_3	OH	$CH(CH_3)_2$	H	CH_3
J	CH_3	OH	CH_2CH_3	CH_3CH_2	OH

FIGURE 3: Chemical structures of cyclosporins A-J

3.1.3.0 **Mechanism of action**

In the course of the developement of immunosuppressive therapy, first generation immunosuppressives (e.g. cyclophsophamide, azathioprine, and methotrexate) were indiscriminately cytotoxic to all dividing cells while second generation therapies (e.g. antilymphocyte serum, total lymphoid irradiation and corticosteroids such as prednisone) act against all immunocompetent cells (12,13,32,44). This in effect left patients open to the possibilities of incurring life-threatening opportunistic infections without the ability to mount a competent immune response. The discovery and introduction of CyA have improved the toxicological implications of immunosuppression.

On a molecular level the mechanism of action of CyA has still not been completely elucidated. However, the importance of this drug stems from the fact that it selectively inhibits subclasses of lymphocytes (T-cells) through the prevention of manufacture or release of interleukin-2. This has the effect of inhibiting activated T-helper cells which in turn blocks the activation of resting T-killer cells (28,45-48). It is important to note that this type of suppression does not completely disable the body's ability to mount an immune response. In addition, the effects of CyA are reversible upon withdrawal of the drug, that is, it is not lymphocytotoxic. Furthermore, CyA is not associated with any myelotoxicity.

Major problems with toxicity stem from nephrotoxic and hepatotoxic effects as well as gum hyperplasia, tremor, and hirsutism. All of these side effects are reversible upon discontinuation of CyA therapy, and are usually attributed to levels above those necessary for therapeutic effect (49,50). Problems with respect to dosage have arisen due to significant inter-individual pharmacokinetic variation causing difficulty in controlling blood levels in the abscence of individual patient monitoring (46). A further complication is that the therapeutic range is poorly defined (14,51).

3.1.4.0 **Commercial production of cyclosporine**

CyA was first purified by Rueger in 1973 (50) and the structure determined in 1976 (52,53). It was not until 1980 that Wenger was able to synthesize the complete molecule (54-58). The complexity of this synthesis makes it impractical to mass produce CyA in this manner and for this reason Sandoz, the commercial manufacturer of this product, uses a microbial fermentation involving submerged cultures of T. inflatum Gams (or a closely related strain). This then necessitates the extraction of the desired product from the broth with an organic solvent and separation of the product from undesirable byproducts (since a range of similar cyclosporins has been identified as natural products of this organism).

Production steps are easily monitored by established HPLC methods (33-43,59-61).

3.2.0.0 Methods for the biosynthesis of cyclosporine

This project consists of two experimental protocols. Section 3.2.1.0 describes the initial screening of five members of the genus Tolypocladium in submerged liquid culture for the organism showing the most potential for large-scale production of CyA. This is followed by section 3.2.2.0 which describes the scaled-up conditions for the cultivation of the chosen production organism in air lift fermentation reactors along with the methods for extraction and purification of CyA from the resulting fermentation broth.

3.2.1.0 Screen for production organism

3.2.1.1 Organisms

Cultures of five different organisms, each belonging to the genus Tolypocladium were obtained from the University of Alberta Mold Herbarium. Their classification and original sources are as reported in Table 2.

Each organism was immediately inoculated onto maintenance slants of cereal agar which then served as a source for working cultures as outlined in Section 2.2.6.0.

Table 2: Organisms for cyclosporine production screen

Organism	UAMH#	Source
T. inflatum	2472	water sample, Whitehorse General Hospital, May, 1965 by J.W.C.
T. inflatum	2880	soil under Pinus contorta "C" horizon Nov. 1967, Kananaskis, Alberta (Paul Widden U of C) received from G.C. Bhatt U of C as Paecilomyces #a77
T. inflatum	4002	muskeg soil at Tuktoyuktuk, N.W.T. by Steve Davies, Micro. U of A, as Tolypocladium inflatum 46 H-46-F, ATCC 38656 Aug. 1976 isolated on mineral salts medium overlaid with crude oil
T. cylindrosporum	4561	mosquito in treehole (mosquito pathogen) at California, received April, 1982 from Mark Goettel, Dept. of Entomology, U of A
T. geodes	4591	in soil, predominantly white spruce woods, Nov. 1976, 30 km SSE of Sudbury, Ontario by A. Carter, received from Mark Goettel, Dept. of Entomology, U of A as DAOM 176470.

3.2.1.2 **Fermentation conditions**

Working media for phase I and phase II cultures consisted of Basic B broth which contained the following ingredients per liter of distilled water: dextrose 40g; casamino acids 10g, $MgSO_4$ .7 H_2O 0.5 g; KH_2PO_4 2.5g; $NaNO_3$ 5.0g; NaCl 5.0g; $FeSO_4$ .7 H_2O 10 mg; (based upon the media used by Drefuss et. al. (17)). pH was adjusted to 6.0.

3.2.1.3 **Sampling procedures**

Fermentation cultures were sampled once each day for a period of 14 days by the withdrawl of 1 ml aliquots of broth. The pH of each sample was measured and samples were subsequently assayed for D-glucose content and antifungal activity. At the end of the incubation period, the remaining broth was extracted twice with an equal volume of ethyl acetate or, in one case with T. inflatum UAMH 2880, one batch was divided into two equal parts, one part being filtered to remove the cells and the other homogenized in a Waring blender, whereupon extraction was carried out on broth alone or on the broth and homogenized cells together. The two organic layers were then dried with $MgSO_4$, transferred to round-bottom flasks, evaporated *in vacuo* on a rotary evaporator to an oily residue, transferred to glass vials and stored under nitrogen at 4°C in preparation for TLC analysis.

3.2.1.4. **Analytical protocols**

D-glucose concentrations were followed using a Glucostat assay kit.

Bioassays were carried out by placing a paper indicator disc (0.25 inch diameter) saturated with broth or extract on the surface of a plate containing Trypticase Soy Agar impregnated with a 10% innoculation of Aspergillus niger

homogenate, incubating the culture plates for 48-72 hours at 27°C and measuring the diameter of any zone of inhibition observed. This assay was carried out before an HPLC system became available. Evidence of antifungal activity was considered as a possible indicator of the presence of cyclosporines which have been reported to have a narrow spectrum of antifungal activity (27,28,30).

TLC analysis was conducted by solubilizing authentic CyA, broth extract, and a mixture of authentic CyA and broth extract in methanol. Each of these solutions was then spotted onto plastic-backed analytical TLC silica gel plate and developed in one of 5 different solvent systems (Table 3, page 41). This was repeated for each of the other four solvent systems. Detection was accomplished by staining the plates with iodine vapor (17).

3.2.2.0 Preparative-scale production of cyclosporine

3.2.2.1 Organism

The microorganism chosen for preparative biosynthesis was Tolypocladium inflatum UAMH 2880, based upon its growth and production characteristics in the screening experiments.

3.2.2.2. Fermentation conditions

Working media for the large-scale production of CyA was designated as 'production media' which was identical to the basic B media used in the screening experiments, with the addition of 5.0 g/L of KCl. The increase in electrolyte concentration has the effect of improving the aeration in the airlift reactors by inhibiting bubble coalescence. This keeps the bubbles small enough to avoid a chugging effect which can cause broth to overflow the reactor vessel. It also promotes a smoother flow and mixing as the broth circulates through the reactor. There was no apparent detrimental effect on the growth of the organism or production of CyA as a result of this increased electrolyte concentration.

The scale-up of the fermentation was carried out by employing a 4.5 L airlift bioreactor as a fermentation vessel. This all-glass vessel was autoclaved as its 4

component pieces (Fig. 2, page 23) and aseptically assembled within a temperature-controlled cabinet. Proportions of inoculum were maintained as in the screening experiment (Section 3.2.1.0).

The large scale fermentation was carried out over a period of 14 days.

3.2.2.3 **Sampling procedures**

Daily samples were collected by removing 3 ml aliquots of broth and by removing all of the metastable foam which had accumulated at the top of the exhaust stack over the previous 24 hour period. Broth samples were then examined for pH, D-glucose, and CyA content (via HPLC analysis), and foam samples were analyzed for CyA content. At the end of the 14th day, the mycelial cells were filtered out of the broth, washed with distilled water and discarded. The aqueous washings were then combined with the broth and extracted as described in Section 3.2.1.3. In the case of the foam, the residue was thoroughly rinsed with a solution of 90% methanol : 10% distilled water and the wash was collected and evaporated *in vacuo* on a rotory evaporator to remove the methanol. The aqueous solution which remained was then extracted two times with an equal volume of ethyl acetate. The ethyl acetate washings were then pooled, dried with $MgSO_4$, and evaporated *in vacuo* on a rotory evaporator to an

oil residue which was sealed in a glass vial under nitrogen and stored at 4°C until analysis.

3.2.2.4 **Analytical protocols**

TLC was carried out under the same conditions as those described in Section 3.2.1.4.

HPLC conditions were: mobile phase 70% acetonitrile : 30% water, flow rate 1 ml/min, detector wavelength 205 nm, and oven temperature 55°C. Injections of 25 ul portions of concentrated broth extract reconstituted in mobile phase onto the HPLC column were conducted serially, using as low a dilution as possible without exceeding the maximum capacity of the column. Collection and pooling of similar fractions of eluent, containing the component with a peak retention time corresponding to that of authentic CyA, were carried out following each injection in order to obtain enough material to make NMR analysis of the product possible. The eluted mobile phase was evaporated <u>in vacuo</u> to dryness using a Savant Speed Vacuum Concentrator/Evaporator with the addition of 0.25 ml portions of methanol to aid in the complete evaporation of the aqueous portion of the mobile phase. A fine white crystalline precipitate was recovered.

This precipitate was dissolved in $CDCl_3$ in preparation for ^1H-NMR spectroscopy.

3.3.0.0 Results and discussion on the biosynthesis of cyclosporine

3.3.1.0 Results and discussion on screen for production organism

Bioassays of extracts from the micro-organism screens indicated that each organism except T. cylindrosporum produced at least one agent which possessed some degree of antifungal activity. Given that CyA is known to have a narrow spectrum of antifungal activity and exhibits antifungal activity against A. niger in our bioassay, T. cylindrosporum was therefore ruled out for further consideration as a possible production organism.

Upon TLC analysis using several different mobile phases, extracts from incubates of each of the remaining organisms were found to contain a metabolite which had an Rf value corresponding to that of authentic cyclosporine (Table 3).

Empirical observation of the intensity of spots observed in the TLC analysis when comparing extracts of cell-free broth with extracts of culture homogenates seemed to indicate that a significant amount of metabolite remained cell-associated when cultures were incubated in shake-flasks. Bioassays indicated that extracts from T. inflatum UAMH 2880 produced the largest zones of inhibition against A. niger.

T. inflatum UAMH 2880 was chosen as the production organism for scale-up to preparative synthesis of CyA because it exhibited the best morphology, growth characteristics (with respect to its ability to grow rapidly in our working medium, in a submerged state, in the form of homogeneous mycelial pellets) and production levels of the desired metabolite.

Table 3: Rf values from TLC of authentic CyA and metabolites from screen organisms. *T. inflatum* UAMH 2880 (A), *T. inflatum* UAMH 4002 (B), *T. inflatum* UAMH 2472 (C), *T. geodes* UAMH 4591 (D).

Mobile Phase	CyA	A	CyA	B	CyA	C	CyA	D
ethyl acetate	0.58	0.62	0.56	0.56	0.55	0.57	0.67	0.64
ethyl acetate, hexane (70:30)	0.20	0.23	0,37	0.37	0.28	0.30	0.33	0.36
ethyl acetate, isopropanol (95:5)	0.86	0.89	0.87	0.91	0.84	0.86	0.85	0.82
benzene, isopropanol (90:10)	0.72	0.72	0.74	0.75	0.73	0.69	0.69	0.71
ethyl acetate, ethyl ether (50:50)	0.41	0.43						

3.3.2.0 Results and discussion on preparative-scale biosynthesis of cyclosporine

During the 15-day fermentation, the pH of the broth gradually dropped from 6.0 to 4.5. The concentration of D-glucose dropped rapidly, after a three day lag period, and was completely consumed by day 13. Using the conditions for HPLC analysis outlined in Section 3.2.2.4, authentic CyA was found to have a retention time of 14.2 minutes (Fig. 4). Upon analysis of the extracts from mycelia, cell-free broth, and foam, all were found to contain a component with a peak retention time identical to that of authentic CyA. It was interesting that the largest concentration of this metabolite occurred in the extract from the foam samples collected from the top of the fermentation vessel during the incubation. In addition, this component appeared to be predominant in the foam extract (Fig. 5), which made further purification by collecting the desired fractions of mobile phase eluent from repeated HPLC injections of foam extract a relatively simple process. A shoulder was visible on the leading edge of the CyA peak in Figure 5. This was due to an incompletely resolved component in the broth. The CyA was eventually separated from this component by only collecting fractions from the tail-end of the CyA peak. The purity of the sample obtained by this fractionation was estimated to be 95%, based on peak area measurements obtained from HPLC analysis.

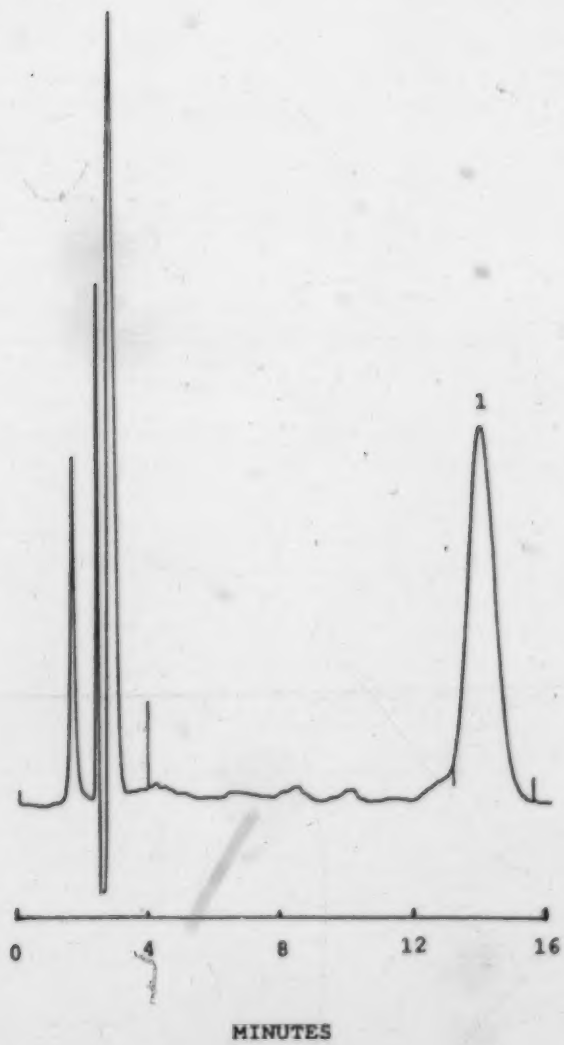


FIGURE 4: High pressure liquid chromatogram of authentic CyA (1).

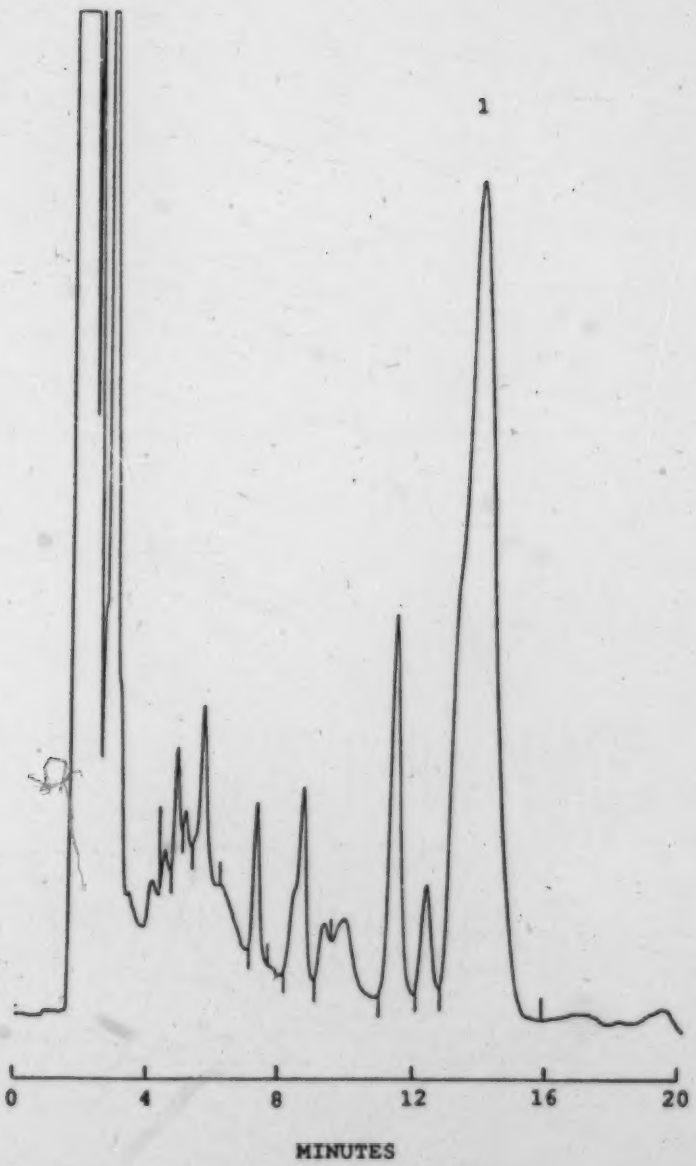


FIGURE 5: High pressure liquid chromatogram of foam extract of T. inflatum UAMH 2880 culture (1 = CyA).

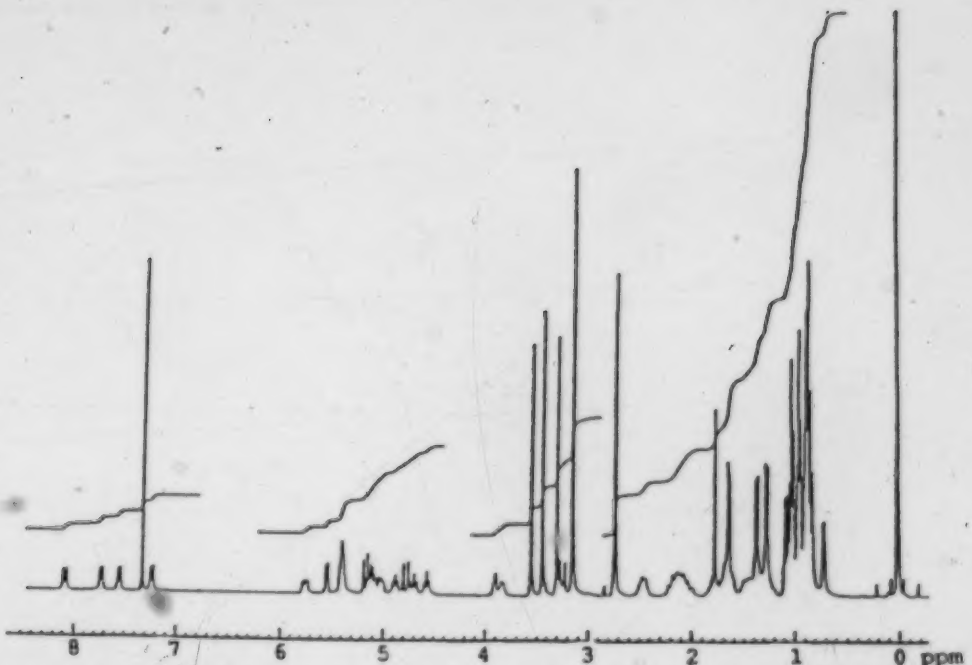


FIGURE 6: 300 MHz ^{1}H-NMR spectrum of authentic CyA (in $CDCl_3$).

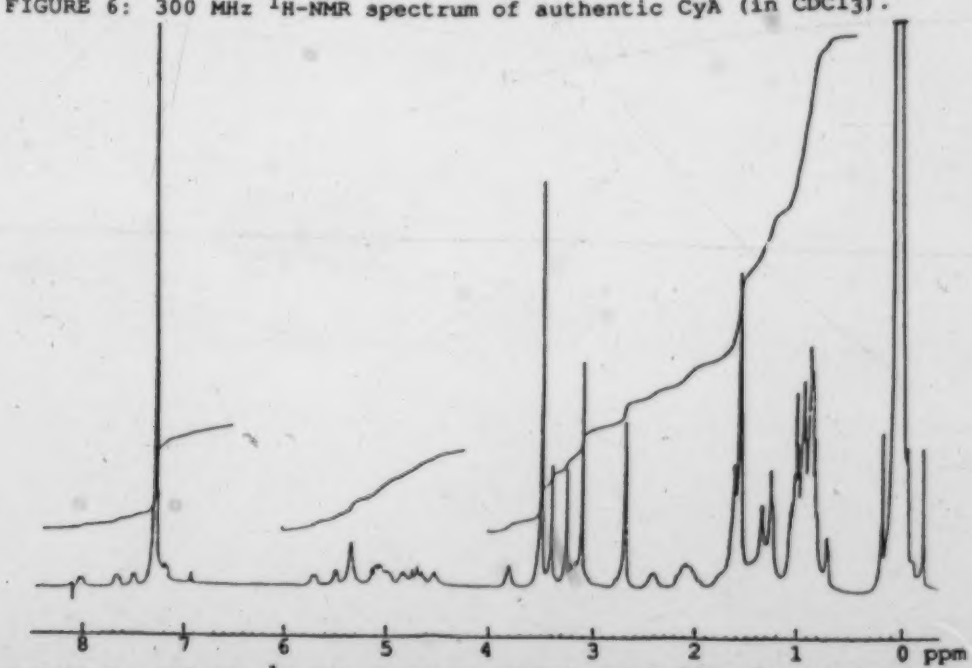


FIGURE 7: 300 MHz ^{1}H-NMR spectrum of CyA metabolite from extract of foam from T. inflatum UAMH 2880 culture (in $CDCl_3$).

The 300 MHz ^{1}H-NMR spectrum of this fungal metabolite (dissolved in $CDCl_3$) produces an extremely complex set of chemical shift resonances (Fig. 7) closely resembling those seen in that of authentic CyA (Fig. 6). Assignments were made based upon comparisons with literature reports and authentic standard. At 8.02, 7.65, 7.5, and 7.18 ppm there were four doublets arising from the resonances of the four -CONH- group protons which were coupled to the proton of the adjacent -CHRCO- group. There was a complex series of 13 signals between 4.5 and 5.8 ppm which were due to the 11 protons attached to the alpha carbon of each amino acid residue (which would be coupled to any protons present on the adjacent N or side-chain carbon) and the two -HC=CH protons. One broad peak at 3.8 ppm was consistent with the presence of an OH group in the R1 position (Fig. 3, page 29), the poor resolution being due to rapid chemical exchange of the hydroxyl proton. Slower exchange would be expected to reveal a doublet due to coupling with the adjacent CH group (compare with Fig. 6). Singlets at 3.5, 3.4, 3.26, two at 3.1 and two more at 2.8 ppm were consistent with the presence of seven -CON(CH_3)- groups, however, the additional singlet at 3.5 ppm, integrating for three protons, was likely due to the presence of methanol carried over from the purification procedures (Section 3.2.2.4). The broad doublet at 1.6 ppm was attributed to the resonance of the methyl protons in the CH_3CH=CH- group. The complex multiplets between 0.5 and 1.7 ppm include the signals from the 16 aliphatic methyl groups.

Comparison of the metabolite spectrum with that of the CyA standard supports an identification of the metabolite as CyA based on the requirement of four CONH groups, seven $CONCH_3$ groups, and a single CHCHOHCH group with the OH at position R1.

In conclusion, we have identified this isolated metabolite of T. inflatum Gams UAMH 2880 as cyclosporine based upon results of its analysis by TLC, HPLC and 1H-NMR which compared favorably with authentic standard and data reported in literature (52). We are now able to apply this method of biosynthesis in order to produce cyclosporine, and other related metabolites, on a preparative scale in order that we might carry out structure and activity studies in the future.

The research project on cyclosporins had two designated objectives at its inception; 1) the biosynthesis of known and novel cyclosporins, and 2) the pharmacological evaluation of these compounds as immunosuppressive agents.

Toward the first objective, we had completed a small screen of filamentous fungi in search of an optimal production organism, and successful scale-up of fermentations from 125 ml culture flasks to a 4.5 L airlift bioreactor provided sufficient material for the structural identification of CyA as described in this thesis.

Other studies, in various stages of completion, were also directed toward attainment of the first objective. This included the use of cell immobilization techniques in order

to carry out the semi-continuous operation of a 4.5 L bioreactor which would help to improve yields, extend culture life and reduce maintenance. It could also potentially take advantage of the fact that the hydrophobic cyclosporin metabolites concentrate into the foam. By discarding the washout media but collecting and extracting batches of foam, a significantly lower volume of solvent would be required. The routine purification of metabolites recovered in this manner was considered to be feasible with the development of a preparative HPLC method.

Another study was an investigation of directed biosynthetic techniques where, by controlling the amounts of amino acids present in the media, the regulation of the production and proportions of known and novel cyclosporins might be achieved.

Further progress in this study required the cooperation of other faculties with regard to the availability of skilled research personnel and access to necessary facilities. Unfortunately suitable arrangements could not be made and it was therefore agreed that work on the cyclosporin project would be suspended at this point.

4.0.0.0 MICROBIAL METABOLISM OF SELECTED DRUGS: LIDOCAINE, MEXILETINE AND FENOPROFEN

4.1.0.0 Introduction to drug metabolism

4.1.1.0 Mammalian drug metabolism

Drugs are often lipid soluble molecules and as such are able to penetrate cell membranes (lipoproteins). The more lipid soluble the drug the more efficiently it can be reabsorbed in the kidney by diffusion into the tubule cells, thus prolonging its residence in the body. The majority of biotransformation reactions have been shown to take place in the liver and are mediated by the complex of membrane-bound enzyme systems embedded in the smooth endoplasmic reticulum of the hepatocytes. This, being a lipoidal environment, partly accounts for evidence that the more lipophilic molecules will tend to be more extensively metabolised given a series of structurally related molecules (63). The end result in all of these reactions is that the drug is made more polar and this aids in the efficiency with which it may be excreted from the body.

Unlike many of the body's other enzyme systems, those concerned with the transformation of drugs, or other non-nutritive potentially toxic xenobiotics, are relatively non-specific. Reactions which they catalyse have been classified

as being of two possible types: phase 1 reactions are those which give rise to oxidized, reduced or hydrolyzed metabolites while phase 2 reactions involve the conjugation of the xenobiotic with some molecule endogenous to the organism. Of the three types of phase 1 reactions in mammals, oxidative biotransformations are quantitatively of greatest importance (6,64). Intensive investigations in this area have identified cytochrome P_{450}-linked mixed function oxygenases as the class of enzymes which catalyze these reactions. Cytochrome P_{450} itself is a class of hemoproteins whose various forms number from at least 20 to possibly as many as 200. Since each isoenzyme has a different substrate specificity, the proportions in which they are present in an individual's liver at a given time will dictate the individual's route(s) of, and capacity for, metabolism of a given drug (65).

4.1.2.0 Microbial models for mammalian metabolism

The obvious preference in studying drug or xenobiotic metabolism would be to have a model which is identical to the target system to which the information garnered will ultimately be applied. In pharmacological studies the human or clinical model is frequently not practical for a variety of reasons ranging from ethical considerations to lack of access to a statistically significant population (e.g., of

individuals who are suffering from a particular disease or are physiologically compromised in some manner), to problems with experimental controls which may require a prohibitive level of individual compliance. This is true especially in preliminary investigations or early on in the development of a drug when acute and chronic toxicity studies are required to help elucidate therapeutic levels, toxic levels and possible side effects. These are a few of the reasons why an alternative model system which closely approximates human metabolism would be a valuable tool in modern metabolic research.

Unfortunately researchers have been unable to develop a model that can adequately mimic human systems in all of the areas required for a truly definitive study. As a result, various in vitro and in vivo models are commonly employed, each with its own shortcomings, but still capable of yielding important information. The value of this information must be qualified by the differences between the model from which it is originally drawn and the system to which it is meant to be applied.

Animal models have been used extensively in metabolic studies but the applicability of data gained from a particular animal model must be evaluated in light of available information on individual and species differences (66). Variables which must be considered include differences in absorption, excretion, distribution, blood levels and active site concentrations (e.g. of a drug and its

metabolites). In addition there may be differences in metabolic pathways (available enzyme systems), rates at which specific transformations occur, as well as external or environmental factors. All of these can have an influence on metabolism in animals and man (66-70).

The scale at which animal studies must be performed can also become an obstacle where the compounds of interest (whether parent drug or metabolites) which are to be separated, detected, and characterized, are present only in trace quantities within a biological sample that contains an extremely complex background matrix of endogenous material. Simple solutions such as increasing the drug dose or increasing the sample size are often impractical due to acute toxic effects poisoning the system or to purely physical constraints with regard to limits of individual size and a finite experimental animal population.

Logistical problems can become prohibitive when working with animals. They are expensive and difficult to obtain, especially larger animals such as the primates which, though difficult to work with, are desirabe because of their close relationship to man. Furthermore, proper maintenance, feeding and housing of these animals in suitable facilities by trained staff is required in order to ensure not only the health of the animals but that of their handlers as well. Even with lower animals, such as rats and mice, costs can accumulate rapidly over a period of time; consider the expense of maintaining a reliable supply of genetically and

microbiologically defined animals and the inclusion of new model types as they are developed.

Finally, the continued and growing lobby against both human and animal experimentation of any kind is increasing political and moral pressure to find and develop suitable alternative models.

One response which addresses some of the problems found in animal models is the recent development of *in vitro* models such as those involving perfused organ systems, cell cultures and isolated microsomal enzyme preparations. These models offer the experimenter a number of advantages including more direct control of many variables which influence the enzyme systems involved in the transformation(s) of interest. In particular, purified cytochrome P_{450} systems would be invaluable as tools for metabolism studies, while specific combinations of different enzymes could allow multi-step transformations to be performed within a single reaction vessel in synthetic processes (19). Unfortunately, the inherent instability of these enzymes *in vitro* due to their dependence on expensive, complex, difficult to maintain system of cofactors and nutrients makes them very difficult to isolate and still retain their activity. Less well defined systems such as microsomal fractions are easier to produce and sustain but the inclusion of additional enzyme systems immediately introduces problems such as competition for substrate and energy sources. This also results in wide variations in the rates and routes of biotransformations

depending upon the relative amounts of competing enzymes present and the nutrients delivered to the system (9,71).

To date, many in vitro models continue to be unstable and technically complex in their operation and they continue to require the sacrifice of lab animals. However, improvements in current technology (e.g., the immobilization of biocatalysts from microbial, plant, animal and human sources) is likely to advance enzyme catalyzed syntheses in the future (19,23,62).

An alternative to these in vitro systems, which has seen increasing popularity in some circles, is the use of microorganisms for preliminary studies in mammalian metabolism. The cytochrome P_{450} monooxygenases in microorganisms have been found to correspond strikingly to certain systems studied in mammals. For instance, bacterial organisms (prokaryotic) resemble extrahepatic cytochrome P_{450} systems such as those found in the adrenal mitochondria, while yeast and fungal organisms (eukaryotic) have cytochrome P_{450} systems which more closely resemble those found in hepatic microsomes (9). As a result, organisms such as Cunninghamella have been proposed as alternative systems for the study of cytochrome P_{450} metabolism with the promise of favorable comparisons between eukaryotic microbial and mammalian hepatic oxidative systems.

Given that the most important hepatic cytochrome P_{450} transformations are oxidative (aided by high blood flow and therefore avaliable molecular oxygen) and that microbial

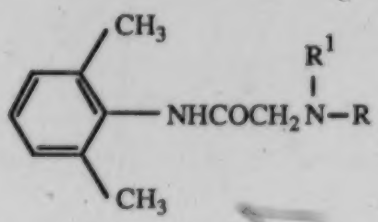
(fungal or yeast) cytochrome P_{450} exhibits similar transformations, if a microorganism or a mix of microorganisms can mimic the functions observed in mammalian metabolism of a xenobiotic, then this could provide a model system which answers many of the problems encountered in animal and *in vitro* models. The large range of organisms avaliable (9), at minimal cost, allows for a wide range of flexibility and specificity in the transformation properties incorporated into a fermentation culture. These cultures are easy to store and maintain requiring very little handling when compared to animals and the fermentation reactions themselves can be viable over relatively long periods of time (months). Nutrients are generally inexpensive and can be easily tied to availability, or perhaps more importantly to optimizing the production, detection and isolation of desired metabolites. This can be extremely valuable since, in general, there are less interfering substances from microbial cells than are found in animal samples. If the media can be simplified to a point where it consists of little more than a buffer and the substrate to be transformed, ease of isolation of the metabolites is greatly enhanced. The versatility of a microbial system in carrying out initial metabolic screens, its facile scale-up to bench-top production of metabolites, and the relatively simple recovery of product from the fermentation broth provides a valuable method for carrying out preliminary studies on the metabolism of a variety of drugs and xenobiotics.

4.1.3.0 Mammalian metabolism of selected drugs:

4.1.3.1 Lidocaine

Lidocaine (Fig. 8) is a type Ib antiarrhythmic agent (reduces maximum rate of depolarization, shortening the action potential duration) and is also a clinically useful local anesthetic (72). The primary mechanism of action is to reduce the action potential duration of the fast sodium channels (which are tetrodotoxin-sensitive), blocking these channels when in its ionic form, possibly by affecting an intracellular receptor site (73). Although it is well absorbed orally, extensive first pass metabolism allows only about 30% of the dose to reach the systemic circulation unchanged and it is therefore limited to IV administration.

Extensive metabolism takes place in the liver, mediated by hepatic cytochrome P_{450} enzyme systems (68,69,74,75). The three different phase 1 reactions which have been observed to take place in mammals (68,70,75-78) are oxidation of the aromatic ring (3- or 4-hydroxylation depending upon the individual species), N-dealkylation, and amide hydrolysis. In man, 72.6% of a dose of lidocaine has been shown to be excreted in the urine as a single major metabolite, 4-hydroxy-2,6-xylidine (68).



	R	R^1
LIDO	CH_2CH_3	CH_2CH_3
MEGX	H	CH_2CH_3
GX	H	H

FIGURE 8: Structures of lidocaine (LIDO) and its active metabolites MEGX and GX.

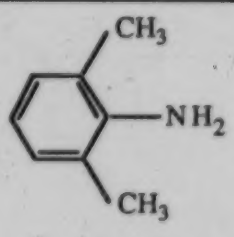


FIGURE 9: Structure of 2,6-xylidine (2,6-XYL)

Two of the resulting metabolites, monoethylglycinexylidide (MEGX) and glycinexylidide (GX) (Fig. 8), also have activity and have been proposed as being responsible for observed side effects when levels of lidocaine were observed to be within the therapeutic range (74,79,80). Major toxic side effects possible with the administration of lidocaine include confusion, slurred speech, diplopia, tremor, severe nausea and vomiting, sinus bradycardia/arrest and seizure (72,81).

Using *in vitro* and *in vivo* systems, investigations into the cytochrome P_{450} systems responsible for the metabolism of lidocaine in the rat have shown that lidocaine is initially either dealkylated or it is hydroxylated to give the primary metabolites MEGX or 3-hydroxylidocaine (3-OH-LIDO) respectively (77,78) (Fig. 10). Any other metabolites, with the exception of 2,6-xylidine (2,6-XYL) (Fig. 9), are most likely produced subsequently through the further transformation of these primary metabolites.

Both the dealkylation and the hydroxylation transformations are catalyzed by cytochrome P_{450} but in each case a different cytochrome P_{450} species or active site is responsible. Evidence that these proceed independently are supported by the fact that changes in pH, ranging from 6.0 to 10.0, and concentration of $MgCl_2$, from 0 to 20 x 10^{-3} M, effects only de-ethylation (69). It has also been shown that the hydroxylating cytochrome P_{450} system has a higher affinity for substrate than the dealkylating one. At low levels of

substrate, the relative amount of hydroxylidocaine produced is increased. However the hydroxylating cytochrome P_{450} system is saturable and as levels of substrate increase, so does the quantity of MEGX produced, to a point where it becomes the predominant metabolite.

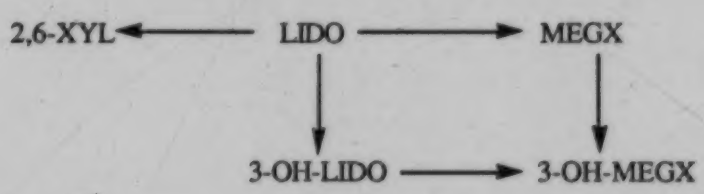


FIGURE 10: Primary oxidative metabolism of lidocaine in rats.

The metabolic pathway of lidocaine has also been shown to exhibit inhibition where secondary transformations of the two primary metabolites (hydroxylation of MEGX or dealkylation of hydroxylidocaine) combine to form 3-OH-MEGX. This secondary hydroxylation of MEGX parallels that of the primary hydroxylation of lidocaine as the dealkylation of hydroxylidocaine parallels the dealkylation of lidocaine. Moreover, the respective parallel transformations are catalyzed by the identical hydroxylating or dealkylating cytochrome P_{450}. In each case, competitive inhibition has been

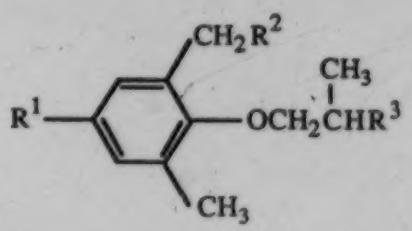
shown to occur (77), that is, high levels of the precursor (LIDO) competitively inhibit the respective secondary transformations, while at the same time high levels of either of the primary metabolites (MEGX or 3OH-LIDO) competitively inhibit the parallel primary transformation of the precursor. The hydroxylating site has also been shown to have a higher affinity for lidocaine than for MEGX. As a result, lidocaine inhibits the hydroxylation of MEGX more than MEGX inhibits the hydroxylation of lidocaine (77).

The multiplicity of the cytochrome P_{450} species which catalyze the various oxidative biotransformations has been considered to be the basic mechanism underlying age, sex and species differences in metabolism, that is, the capacity of an individual organism to produce different levels of a variety of metabolic products is based upon the ratio of the different species of cytochrome P_{450} present at that time (67-70,77,78).

These reports of the saturability of cytochrome P_{450} sites which catalyze hydroxylation (at higher levels of lidocaine) together with the demonstration of substrate inhibition help to explain why the metabolism of lidocaine appears to be dose-dependant and how levels of MEGX, and therefore GX, can, under certain circumstances, accumulate in some individuals to a point where their level of activity takes on therapeutic significance.

4.1.3.2 **Mexiletine**

Mexiletine (Fig. 11) is also a type Ib antiarrhythmic and as such resembles lidocaine in its electrophysiological effects. Originally investigated for its anticonvulsant properties, it was also found to have a strong local anesthetic effect equal to that of lidocaine (82). Unlike lidocaine, mexiletine does not undergo as extensive first pass metabolism and can therefore be administered orally. It also has a longer half-life. Unfortunately, mexiletine has a high incidence of side effects which severely limits its use (83,84). Metabolism takes place largely in the liver, less than 15% of the parent drug is excreted unchanged (85). Eight metabolites of mexiletine have been identified (85-88). The four major metabolites include hydroxymethylmexiletine, *para*-hydroxymexiletine, and their corresponding alcohols (Fig. 11). None of these metabolites demonstrates significant activity (83).



	R^1	R^2	R^3
Mexiletine	H	H	NH_2
Para-hydroxymexiletine	OH	H	NH_2
Hydroxymethylmexiletine	H	OH	NH_2
1-(4'-hydroxy,2',6'-dimethyl) phenoxypropan-2-ol	OH	H	OH
1-(2'-hydroxymethyl, 6'-methyl) phenoxypropan-2-ol	H	OH	OH

FIGURE 11: Structure of mexiletine and four of its major metabolites

4.1.3.3 **Fenoprofen**

Fenoprofen (Fig. 12) is a chiral molecule which belongs to the 2-arylpropionic subgroup (also known as the "profens") of the non-steroidal anti-inflammatory drugs (NSAIDs). Fenoprofen has been shown to possess anti-inflammatory and analgesic effects in both animals and man (89-92). Fenoprofen is adminstered orally, the calcium salt being the most favorable preparation. It is rapidly and efficiently absorbed from the gastrointestinal tract and has a half-life of approximately 2.5 hours.

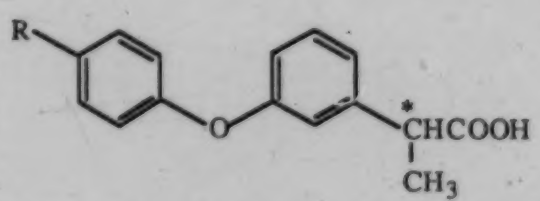


FIGURE 12: Structures of fenoprofen (R = H), 4'-hydroxyfenoprofen (R = OH), and 4'-methoxyfenoprofen (R = OCH_3)

Based upon urinary recovery of fenoprofen and metabolites, at least 85% of a 250 mg dose is absorbed and excreted, and less than 2% of the dose appears in faeces

collected over 48-72 hours. Over 90% of the dose is metabolized (presumably in the liver). Evidence suggests fenoprofen undergoes tubular reabsorption by the kidneys, but no metabolites have been observed in plasma (93,94).

Metabolites have been reported to consist mainly of glucuronide conjugates of fenoprofen and 4'-hydroxyfenoprofen. Small amounts of unchanged fenoprofen, 4'-hydroxyfenoprofen, and unidentified conjugates of fenoprofen and 4'-hydroxyfenoprofen have also been found excreted in the urine (95).

Another aspect of fenoprofen which has pharmacologic implications is the fact that it possesses a chiral center (designated * in Fig. 12). Activity has been shown to reside almost entirely in the sinister (S) enantiomer (which is generally dextrorotary in NSAIDs), while little or none is seen with the rectus (R) enantiomer (which is generally levorotary) (96,97). Administered as a racemic mixture (as are all profen-type NSAIDs excluding naproxen which is marketed as (S)-naproxen), fenoprofen is known to undergo metabolic chiral inversion of the assymmetric center. It is important to note that this inversion is unidirectional, i.e. from (R) to (S), and involves no other metabolic transformation of the compound (96,97). It is also noteworthy that stereochemistry is known to influence the rates of active absorption, distribution, and elimination of a drug as well as the rate and route of its metabolism (18). In the case of NSAIDs, the inactive (R) enantiomer is generally more

rapidly eliminated (having a shorter plasma half-life and smaller area under the concentration-time curve) than the (S) enantiomer (97). These differences may be due to stereoselectivity in distribution and/or elimination or even in stereoselective metabolism which does not involve the chiral center directly (e.g., aryl oxidation).

Problems may arise from administering the racemic drug because of the different rates at which individuals carry out the inversion. This, in turn, may cause inter-individual differences in clinical effect for an apparently therapeutic plasma concentration of the drug (96,97). Take, for example, the administration of a racemate to two different individuals, and only the (S) enantiomer is active. When a stereospecific assay is not used, evidence correlating plasma concentration of the racemate with clinical effect can be misleading. If the first individual is capable of rapid inversion 50% of the dose administered, the (R) enantiomer, can be considered analagous to a prodrug and is available to exert clinical effect. A second individual, given a similar dose, carrying out this inversion at a slow rate will exhibit an apparent decrease in activity because in this instance the inactive (R) enantiomer is excreted before appreciable conversion to the active (S) enantiomer, i.e., the (R) enantiomer exists as an impurity, cutting the effective dose in half).

Under such conditions a stereospecific assay would be invaluable in therapeutic monitoring of active drug levels.

The administration of pure (S) enantiomer could also be beneficial in that it would: a) reduce the dose of total drug, lessening the chances of attaining toxic levels of drug due to non-stereospecific mechanisms, i.e., where both the active and the inactive enantiomers contribute to toxicity, b) it would decrease variability in therapeutic response arising from variability in individual rates and extent of inversion, and c) it would decrease variability in toxic response due to stereospecific mechanisms arising from variability in individual rates and extent of inversion. (Toxicity of NSAIDs to the gastrointestinal tract and actions on blood clotting arise from the same mechanisms as their anti-inflammatory actions, i.e., through the inhibition of prostaglandin biosynthesis, therefore, chiral inversion of these drugs would likely enhance their toxicity (96).)

In the case of fenoprofen, after administration of the racemate, plasma ratios (S/R) show a predominance of (S)-fenoprofen. A rapid drop in the (R)-fenoprofen is observed until it becomes undetectable after about 4 hours (250 mg dose) (94).

In the analysis of enzyme hydrolyzed urine,(95) (R)-fenoprofen was not detected at all when patients received a 600 mg dose while less than 4% of a 1200 mg dose was recovered as (R)-fenoprofen. Overall, 37-50% of a dose was recovered as fenoprofen, while (S)-4'-hydroxyfenoprofen accounted for "most of the rest". The only other metabolites

reported were minor amounts of unidentified conjugates of fenoprofen and 4'-hydroxy-fenoprofen.

The fact that both (S)-4'-hydroxy-fenoprofen and (R)-4'-hydroxy-fenoprofen (even though in small amounts) were detected in urine suggests that both the (S) and the (R) enantiomers of fenoprofen undergo hydroxylation.

Since the quantities of (S)-fenoprofen and (S)-4'hydroxyfenoprofen recovered in the urine have been shown to account for 78-86% of the total dose of the racemic mixture, stereoselective biotransformation of (R)-fenoprofen to (S)-fenoprofen must therefore occur. (This does not exclude the possibility of preferential absorption and/or excretion of (S)-fenoprofen or (R)-fenoprofen as well, the unequivocal confirmation of these occurring can only be resolved with the administration of the pure enantiomers.)

4.1.4.0 **Choice of microorganism**

There is ample evidence in the literature documenting the high capacity of filamentous fungi to metabolize xenobiotics (2,5,9,98-105). We therefore began investigations with several species (Cunninghamella, Aspergillus, and Rhizopus) known to be capable of catalyzing aliphatic and aromatic hydroxylations, C-oxidation, N-dealkylation, O-dealkylation, N-oxidation, glucosylation, esterification, and acetylation. Ongoing investigations utilized the organism which offered the most promising results. C. enchinulata in

particular has been shown to closely parallel a variety of well known hepatic oxidative transformations found in mammals, and is able to produce several metabolites of drugs and xenobiotics of interest in high yields (8,106-113).

4.2.0.0 Methods for the microbial metabolism of lidocaine, mexiletine and fenoprofen

4.2.1.0 Screen of organisms

The following five organisms, all filamentous fungi, were screened for their ability to metabolize lidocaine or mexiletine: Cunninghamella echinulata UAMH 4144, Cunninghamella echinulata UAMH 4145, Aspergillus niger UAMH 1240, Aspergillus flavus UAMH 2679, and Rhizopus nigricans. Cunninghamella echinulata UAMH 4145 was also evaluated for its ability to hydroxylate fenoprofen because a previous screen carried out by Dr. N. Singh (a fellow research student) indicated that this might be a major transformation pathway in this organism, indicating a similarity to human metabolism (114).

4.2.1.1 Fermentation conditions

Media used in the screening experiments consisted of Czapek Dox broth for C. echinulata 4144, C. echinulata 4145, A. niger and A. flavus while Tripticase Soy Broth was used for cultivation of R. nigricans because of its poor growth in Czapek Dox Broth.

Phase II cultures were incubated for 24 hours in an environmental shaker-incubator prior to being spiked with a

solution containing 5 mg of substrate dissolved in a minimum amount of distilled water, or 95% ethanol in the case of fenoprofen, which had been sterilized by passage through a 0.22 um filter. Incubations were allowed to proceed for a period of 18 days.

4.2.1.2 Sampling procedures

Phase II fermentation cultures were sampled once each day for a period of 18 days (or until one day after substrate was no longer detectable) by withdrawl of 1 ml aliquots of broth, which were processed as described below.

4.2.1.3 Extraction and derivitization of samples and standards from lidocaine and mexiletine screens

Samples were prepared for GC or GC/MS analysis by conducting a simultaneous aqueous derivatization and extraction (76,115-119) employing pentafluorobenzoyl chloride (PFBC) as the derivitizing agent and toluene as the organic solvent in the following protocol: 1 ml of distilled, deionized water and 200 mg $KHCO_3$ were added to 1 ml aliquots of broth from phase II cultures in screw-cap test tubes and mixed on a vortex mixer until the $KHCO_3$ was completely dissolved. 5 ml of PFBC and 3 ml of toluene were then added, the test tubes were sealed with teflon-lined caps, mixed on a

vortex mixer for 30 minutes, and centrifuged for 5 minutes at 2000 g (Adams Dynac Centrifuge). The organic layer was then transferred to a clean test tube and evaporated to dryness (Savant Speed Vacuum Concentrator/Evaporator). Each residue was then reconstituted in 300 ul of toluene, and transferred to a 1.5 ml microfuge tube to which 600 ul of 5% NaOH was also added. After vortexing for 1 minute, followed by centrifuging in a microfuge (Brinkman Microfuge B) for 1 minute, the aqueous layer was discarded. Extracts were then analysed by GC or GC/MS by injection of 1 ul aliquots of organic layer on column.

Standards were handled in a similar manner, substituting 1 ml of 30 ug/ml aqueous solutions (in water and media) of lidocaine HCl, MEGX HCl, or mexiletine HCl for 1 ml of broth.

N-acetylated mexiletine was prepared for comparison with the proposed microbial metabolite by derivatizing mexiletine in aqueous solution (76,115-119) with acetic anhydride using the following procedure: 2 ml of an aqueous standard solution containing 30 ug/ml of MEGX HCl was adjusted to pH 8.5 by saturating it with a small excess of K_2CO_3. After the addition of 300 ul of acetic anhydride, the solution was vortexed until it stopped effervescing (making sure that the K_2CO_3 was in excess by maintaining a small amount of undissolved solid K_2CO_3 in the bottom of the tube). The resulting aqueous solution was then extracted by adding 4 mls of toluene, vortexing for 30 minutes in a sealed screw-cap tube and centrifuging at 2000 g for 5 minutes. The organic

layer was collected, evaporated to dryness (Savant Speed Vacuum Concentrator/Evaporator), and the residue was reconstituted in 100 ul of toluene in preparation for analysis by GC and GC/MS.

4.2.1.4 Conditions for GC, GC/MS analysis of lidocaine and mexiletine samples

Conditions for analysis of fermentation broth extracts containing lidocaine or mexiletine and their respective metabolites were as follows. If the analysis was on the DB-1701 column (30 m,0.25 mm ID, 0.25 um film thickness), the oven temperature was programmed from 130°C to 280°C at 8°C/min. The initial temperature was held for 2 minutes. If the column used was the DB-1 (30 m, 0.25 mm ID, 0.25 um film thickness), the temperature gradient was reduced to 4°C/min.

4.2.1.5 Extraction and derivatization of fenoprofen samples

HPLC analysis of samples for fenoprofen and its metabolites was conducted in cooperation with Dr. R. Mehvar using the following methods (120).

For the stereospecific assay: 0.05 ml of internal standard (0.05 mg/ml racemic ketoprofen) and 0.1 ml of sulfuric acid (0.6 M) were added to 0.5 ml of broth. The

constituents were then extracted with 3 ml of a mixture of isooctane and isopropanol (95:5) following vortex-mixing for 30 seconds and centrifuging at 2000 g for 5 minutes. The organic layer was transferred to a clean tube and 2 ml of HPLC-grade water was added. After vortex-mixing for 15 seconds and centrifuging at 2000 g for 3 minutes, the organic layer was discarded. Back-extraction was performed by acidifying the aqueous solution with 0.2 ml 0.6 M sulfuric acid, adding 2.5 ml chloroform, vortex-mixing for 15 seconds and centrifuging at 2000 g for 3 minutes. The organic layer was transferred to a clean test tube and evaporated to dryness. The residue was then reconstituted in 0.1 ml 50 mM TEA in acetonitrile. Solutions of 0.05 ml of ethyl chloroformate and 0.05 ml of L-leucinamide were added in 30 second intervals. Two minutes after the addition of L-leucinamide, 0.05 ml distilled water was added and aliquots of 10 to 40 ul of the solution were injected into the HPLC.

Sample preparation for non-stereoselective fenoprofen analysis consisted of protein precipitation of the sample broth by the addition of an equal volume of acetonitrile followed by vortex mixing for 30 seconds, centrifugation for 3 minutes and injection of 0.01 to 0.04 ml of the supernatant into the HPLC. No derivitization was necessary.

4.2.1.6 Conditions for the HPLC analysis of fenoprofen samples

The wavelength of choice for monitoring fenoprofen and its metabolites was 232 nm. Unfortunately, some endogenous substances, coeluting with the internal standard, also absorbed at 232 nm. Since the internal standard absorbed at 275 nm as well, while the coeluting substances did not, the variable wavelength detector was set at 275 nm initially and after 13 minutes, following the elution of the internal standard, the wavelength was switched to 232 nm for the remainder of the run (120).

The mobile phase for the stereospecific analysis consisted of monobasic potassium phosphate (0.07 M): acetonitrile: TEA (35:65:0.02) with a pH of 6.0. The flow rate was set at 1 ml/min.

The non-stereospecific analysis of fenoprofen was carried out under the same chromatographic conditions described above but with a mobile phase consisting of 0.07 M monopotassium phosphate : acetonitrile : TEA (70:30:0.02).

4.2.2.0 Scale-up of biosynthesis of fenoprofen metabolite

4.2.2.1 Fermentation conditions

10 mg of fenoprofen calcium was dissolved in a minimum amount of 95% ethanol, filter-sterilized and added to a 24 hour phase II culture of C. echinulata UAMH 4145 contained in a 170 ml airlift fermentation vessel.

4.2.2.2 Extraction procedure

After an incubation period of 14 days, the mycelial cells were filtered from the broth, washed with distilled water, and the washings were then pooled with the broth, giving an aqueous solution of about 300 ml. This aqueous solution was acidified to pH 2 with concentrated HCl, saturated with NaCl, and extracted three times with 100 ml portions of an organic solvent containing a mixture of ethyl ether and dichloromethane (3:2). The organic layers were collected, pooled, dried with Na_2SO_4, filtered and evaporated in vacuo on a rotory evaporator to give a brown oil consisting of acidic, amphoteric, and neutral components of the broth. This oily residue was then reconstituted with 40 mls of fresh ether/dichloromethane and extracted three times with 25 ml volumes of an aqueous solution of NaOH of pH 12.

The aqueous layers (containing acidic and amphoteric compounds) were then pooled. This aqueous solution was acidified to pH 2 with concentrated HCl and extracted three times with 50 ml volumes of the ether/dichloromethane. The organic layers were pooled, dried with Na_2SO_4, and evaporated to give a brown oil containing acidic and amphoteric components from the fermentation broth.

4.2.2.3 Purification and analysis of microbial metabolite of fenoprofen

The brown oil obtained from the extraction procedure described above was reconstituted in an equal volume of ether/dicholoromethane (3:2) and streaked on 10 x 20 cm HPTLC plates precoated with a 0.25 mm thick layer of silica gel 60 and containing a preconcentrating zone. After drying, these plates were then developed using a mobile phase of methanol : dicholoromethane (10:90). The plates were dried thoroughly and a portion of one plate was exposed to iodine vapour. The iodine-exposed plate was then used as a template in separating visualized spots by scraping off fractions of silica gel containing individual components. These components were desorbed from the silica by washing with hot methanol. The methanol washes were collected separately, evaporated to dryness and the resulting residual components (from the TLC plates) were analysed by HPLC using the procedures described previously (Section 4.2.1.6). One residual fraction obtained

from the TLC plates was found, upon HPLC analysis, to produce peaks with retention times of 30.5 and 36.4 minutes. These were identical to the predominant peaks observed to accumulate while monitoring broth samples (by HPLC) collected during the course of the fermentation. This residue was then subjected to IR (thin film on a NaCl disk), ^{1}H-NMR (using DMSO-d_6 as the solvent), and high resolution EIMS for structural analysis and was found to be composed of a single compound.

4.2.3.0 Human metabolism of fenoprofen

4.2.3.1 Dosing of subjects and collection of samples

Each of three male subjects was administered a single 300 mg dose of fenoprofen and their urines were collected over a period of 12 hours. The urines were then pooled and basified to pH 12 with NaOH to hydrolyze glucuronide conjugates, then extracted and processed in the manner outlined in Section 4.2.2.2, to give a brown oil.

4.2.3.2 Purification and analysis of human metabolites of fenoprofen

The brown oil extracted from urine was subjected to TLC using the conditions described for the TLC of the microbial extract (Section 4.2.2.3). A portion of one of the TLC plates used in this procedure was exposed to iodine vapor. The spots visualized in this manner were insufficiently resolved to allow separation of all of the components present in the oil extract. Therefore, fractions of silica containing components with an Rf of 0.20 to 0.50 (chosen arbitrarily, authentic fenoprofen having an Rf of 0.36) were scraped from the TLC plates. Components were desorbed from the silica using hot methanol, and the solution was evaporated in vacuo to a brown

oil which was reconstituted in an equal volume of diethyl ether : dicholoromethane (3:2) and subjected to TLC a second time, using a mobile phase of toluene : isopropyl alcohol (85:15). A portion of one of the TLC plates used in this process was exposed to iodine vapor to visualize component spots. Using the iodine-exposed plate as a template, regions of silica containing individual component spots were scraped from the plates. Each spot was then desorbed from the silica using hot methanol, dried in vacuo and analyzed by HPLC using the procedures described in Section 4.2.1.6.

One component spot, a brown oil, was found to give HPLC peak retention times identical to a component of the microbial extract. This material from the urine extract was then subjected to analysis by IR (thin-film), ^{1}H-NMR (using DMSO-d_6 as the solvent), and high resolution EIMS.

4.3.0.0 Results and discussion on microbial metabolism studies

4.3.1.0 Results and discussion on microbial metabolism of lidocaine

Two of the screen organisms, C. echinulata UAMH 4145 and R. nigricans, carried out appreciable metabolism of lidocaine. In both cases these organisms produced the N-dealkylated product MEGX. GC retention time (16.73 min on DB 1701) and mass spectral data (Fig. 13,14) [m/z (% relative abundance): 400(6), 280(17), 252(25), 205(3), 195(100), 167(12), 121(43), 105(10)] of the metabolite derivatized with PFBC were identical to those of the derivatized authentic standard MEGX. Both the metabolite and the authentic standard lost fragments of m/z 195 and m/z 167 corresponding to the loss of $C_6F_5CO^+$ and $C_6F_5^+$ respectively, a characteristic of compounds containing a PFB group. Identification of other potential metabolites suggested by 3 other peaks seen in GC traces of broth extract was prevented due to the inability to resolve those same peaks during GC/MS analysis.

The significance of these microorganism's ability to transform lidocaine into MEGX arises from the fact that this same transformation has been shown to occur in mammals, indicating a similarity in their cytochrome P_{450} systems. In addition, the particular metabolite seen here has been

identified as an active metabolite of lidocaine, which reinforces the possible use of microorganisms in the production of pharmacologically active metabolites for structure and activity studies. In the specific case of MEGX however, this would not apply since it is readily produced through traditional chemical synthetic techniques.

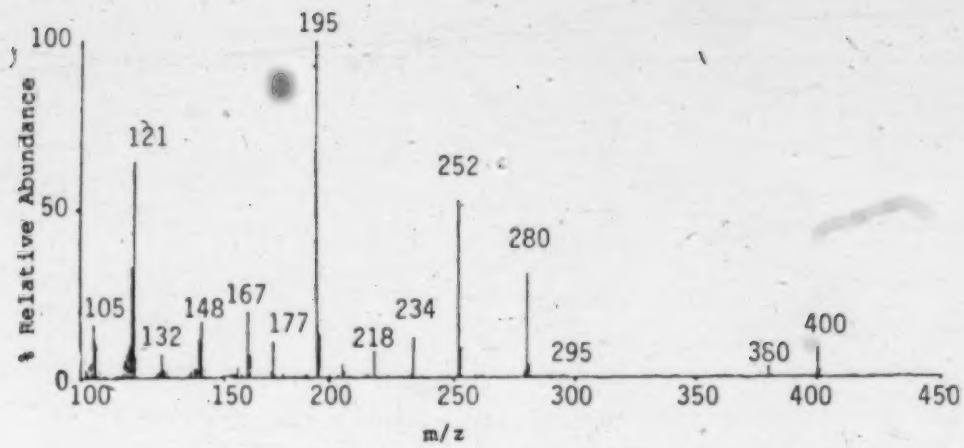


FIGURE 13: EIMS-line diagram of PFB derivative of the proposed microbial N-dealkylated metabolite of lidocaine (MEGX).

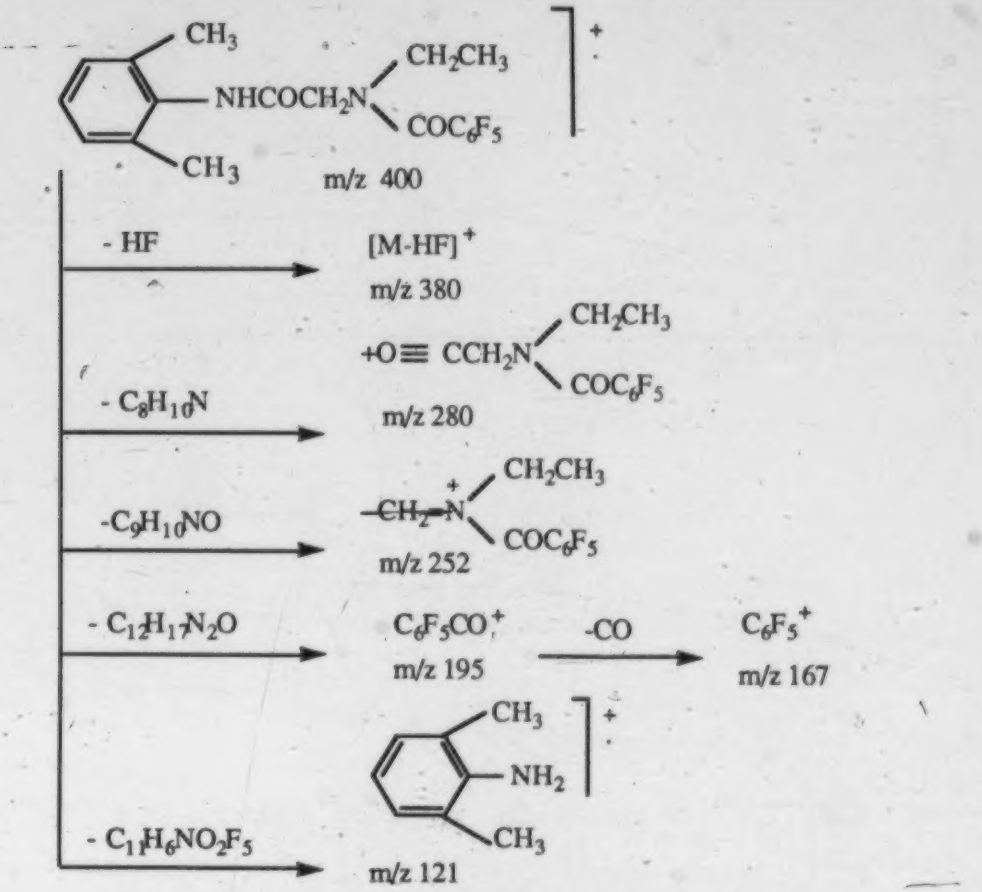


FIGURE 14: Fragmentation pattern of the PFB derivative of the proposed N-dealkylated metabolite of lidocaine (MEGX).

4.3.2.0 Results and discussion on microbial metabolism of mexiletine

Two organisms were found to metabolize significant amounts of mexiletine. Extracts of broth from C. echinulata UAMH 4145 and A. flavus UAMH 2679, derivatized with pentafluorobenzoyl chloride (PFBC), contained a metabolite peak with a GC retention time of 15.36 min on a DB-1701 column that was identified as the N-actetylated metabolite of mexiletine (Fig. 15), based on GC retention time and mass spectral data (Fig. 16) [m/z (% relative abundance): 222(13), 178(7), 162(42), 122 (100), 121(74), 105(88)]. These were identical to those of an authentic mexiletine standard which was derivatized with acetic anhydride in aqueous solution and extracted as outlined in Section 4.2.1.3. This metabolite was not PFB-derivatized, since it failed to produce characteristic fragment ions of m/z 195 and m/z 167 common to compounds which contain a PFB group. The EIMS-line diagram in Fig. 17(a) shows ions at m/z 243 and m/z 259 which are likely impurities in the sample. The apparent presence of a molecular ion at m/z 222 rather than at m/z 221 is unexpected. However, the prominant m+1 ion of m/z 222 in the CIMS-line diagram of the same sample (Fig. 17(b)) would tend to support that the compound present has a molecular weight of 221. The discrepancy in the EIMS-line diagram could be attributed to a slightly out-of-tune instrument.

Although the potential for microbial N-acetylation has been documented (8), this particular transformation of mexiletine is not known to occur in man (85-87).

A second metabolite peak found only in C. echinulata UAMH 4145 culture extracts, was found to have a GC retention time for its PFB derivative of 18.16 min on a DB-1 column. Based upon fragmentation pattern of the PFB derivative during GC/MS analysis (Fig. 17,18), this compound was identified as 1-(2'-hydroxymethyl-6'-methyl)-phenoxy-2-aminopropane, i.e., the hydroxymethyl metabolite of mexiletine [m/z (% relative abundance): 389(3), 252(49), 238(3), 195(100), 167(27), 138(14), 137(11), 121(15)].

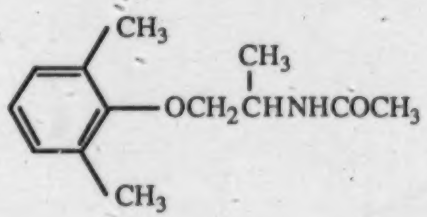


FIGURE 15: Structure of N-acetylmexiletine

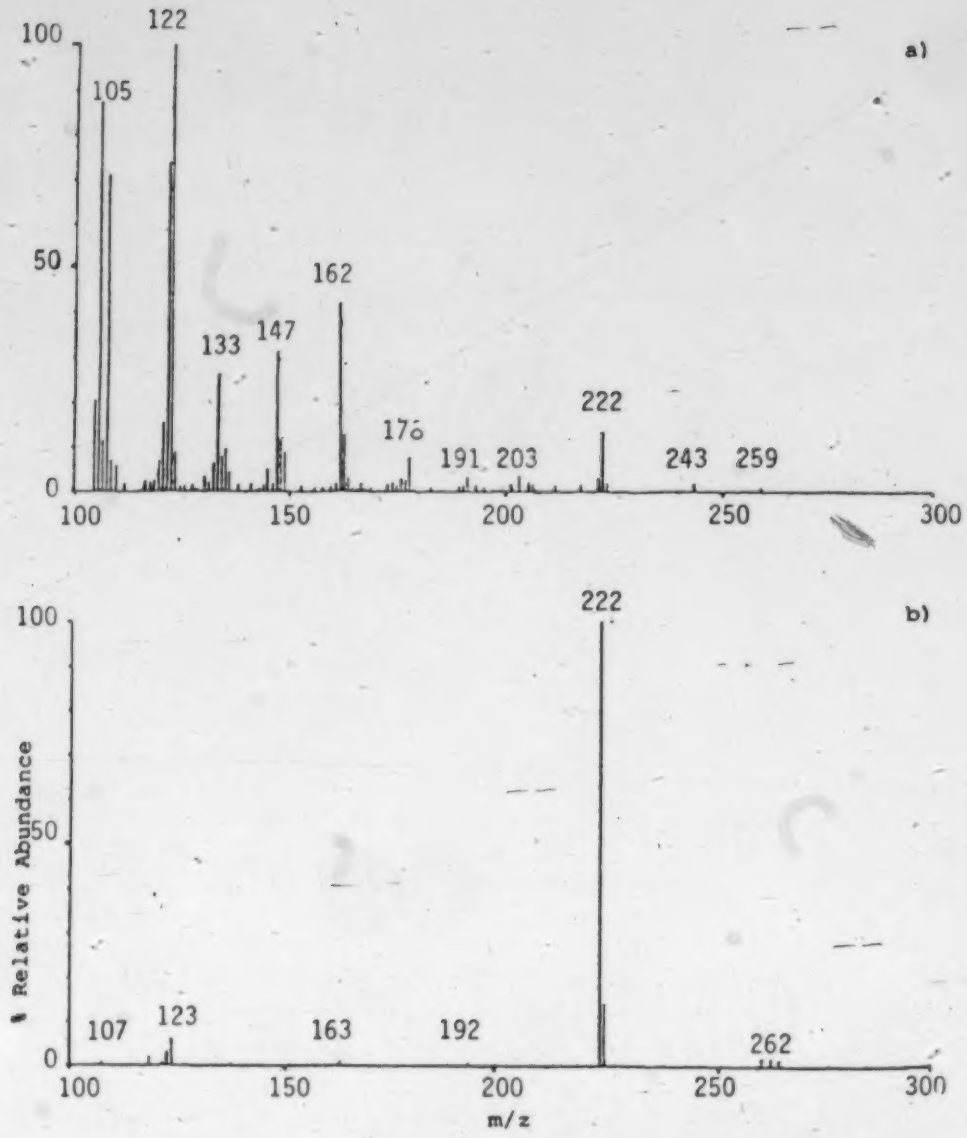


FIGURE 16: a) EIMS and b) CIMS-line diagrams of PFB derivative of the proposed microbial N-acetylated metabolite of mexiletine

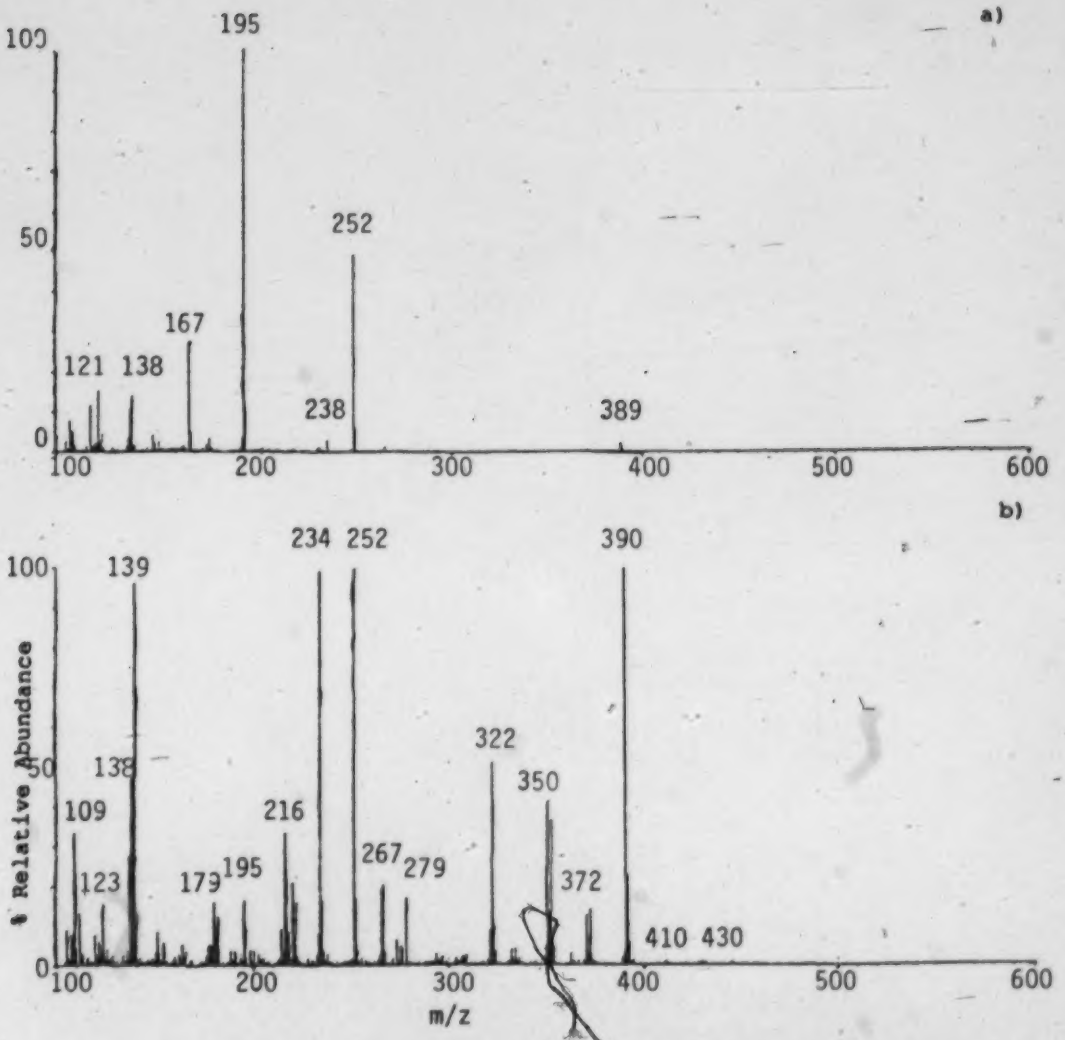


FIGURE 17: a) EIMS and b) CIMS-line diagrams of PFB derivative of the proposed hydroxymethyl metabolite of mexiletine from extract of broth from culture of C. echinulata UAMH 4145.

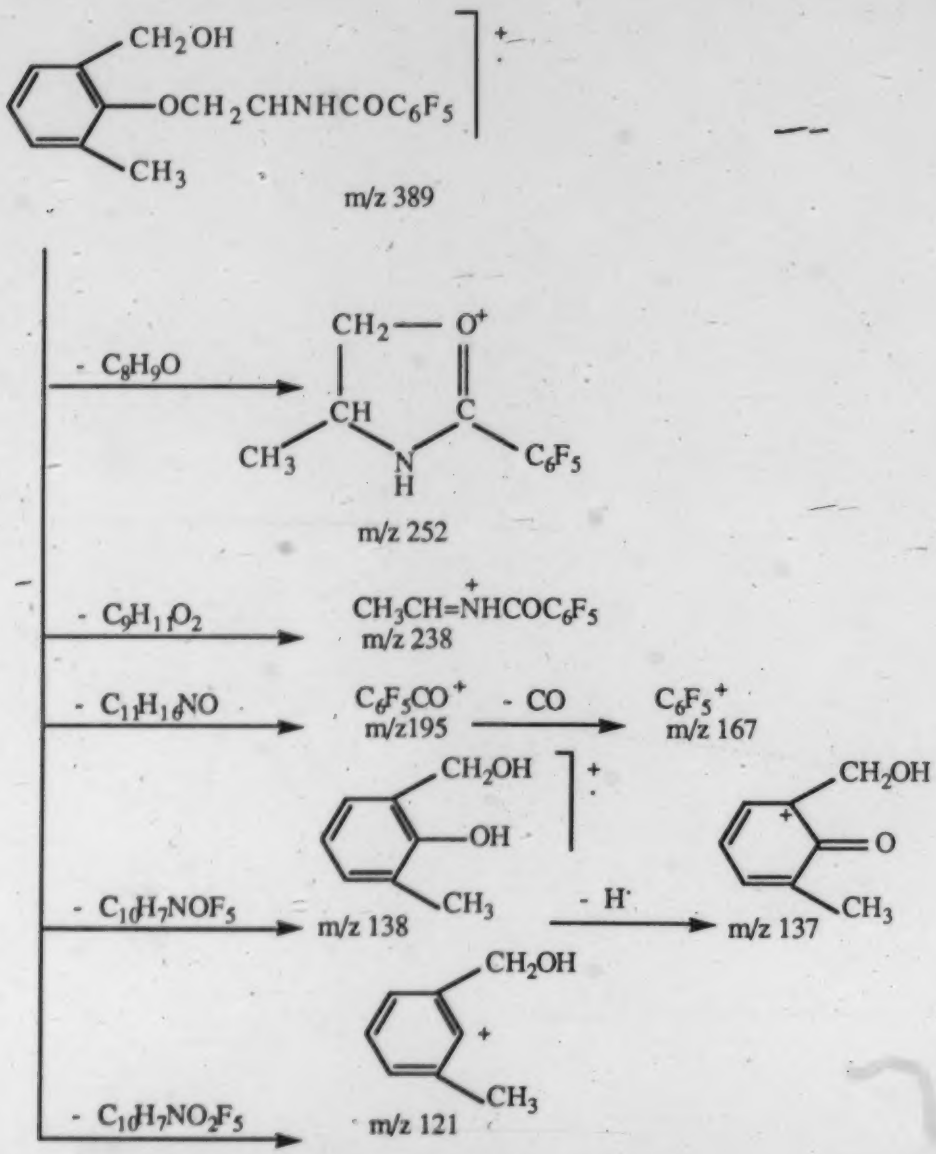


FIGURE 18: Fragmentation pattern of the PFB derivative of the proposed hydroxymethyl metabolite of mexiletine.

The peaks at m/z 195 (base peak) and m/z 167 are characteristic of a PFB derivatized compound fragmenting to produce $(C_6F_5CO)^+$ and $(C_6F_5)^+$ ions respectively. The molecular ion (m/z 389) loses $C_9H_{11}O_2$ to give $(C_9H_5NOF_5)^+$ m/z 238. The site of the oxygen was deduced to be on one of the methyl carbons rather than directly on the ring or in the side chain because the molecular ion loses $C_8H_9O_2$ to give $(C_{10}H_7NOF_5)^+$ indicating that the single oxygen present on the side chain most likely belonged to the PFB group. Ions at m/z 138, 137, and 121 each indicate an oxygen substituted somewhere on or near the ring portion of the molecule. Finally CIMS (with methane) showed a loss of water from (M+1) suggesting a hydroxyl group not bound to the ring.

A third metabolite peak, again from extracts of C. echinulata UAMH 4145, having a retention time of 23.24 min (on a DB-1 column), gave a quasimolecular ion (M+1) of 584 on CIMS and low resolution EIMS data [m/z (% relative abundance): 252(16), 238(4), 195(100), 167(21)] indicating the possible presence of a di-derivatized hydroxyl metabolite of mexiletine. This di-derivatization would imply the presence of a 3- or 4-hydroxylated metabolite and this is supported to some extent by the absence of the loss of water from the quasimolecular ion (CIMS) as seen in the case of the second metabolite. In the absence of authentic standard, the scale-up of the fermentation and purification of larger quantities of metabolite will be required in order to confirm the structure by NMR and IR analysis.

If C. echinulata could be shown to preferentially *para*-hydroxylate mexiletine and similar compounds, paralleling human metabolism, it could be developed as an important organism for the possible production of other similar human metabolites in drug metabolism studies. Confirmation of the structure of these mexiletine metabolites requires the direct comparison of each metabolite's chromatographic characteristics with those of their authentic standards, along with their preparative-scale production in order to carry out the appropriate IR, NMR, and mass spectrometric analyses. However these metabolites of mexiletine are not known to possess significant pharmacological activity, limiting the usefulness of large scale production of these particular metabolites (83).

4.3.3.0 Results and discussion on microbial metabolism of fenoprofen

HPLC analysis of L-leucinamide derivatized standards gave retention times of 19.1 and 22.0 minutes for (R) and (S)-fenoprofen, and of 10.4 and 12.2 minutes for (R) and (S)-ketoprofen respectively. Analysis of samples from the initial screen cultures revealed two additional peaks at 30.5 and 36.4 minutes, no other peaks were detected (Fig. 19). The low degree of attenuation seen in Figure 19 was used in order to give minor peaks better definition. Additional runs were also made where the largest peaks were kept on-scale. The fenoprofen was very slowly metabolized during the incubation, by day 11 over 80% of the parent compound remained and was still far from being metabolized on day 18 when the fermentation was terminated. We were particularly interested in the potential selectivity of the metabolism. If these two peaks were diastereomers of one exclusive metabolite, then there was a possibility that this microbial biotransformation might parallel human biotransformation since man has been reported to produce only one phase 1 metabolite, 4'-hydroxyfenoprofen. In addition, assuming the two peaks represent metabolites of the fenoprofen enantiomers, it is possible that the microorganism metabolizes fenoprofen in a stereoselective manner, since these peaks did not increase in proportion to each other. The later eluting peak had a consistently larger peak area which was approximately 1.5

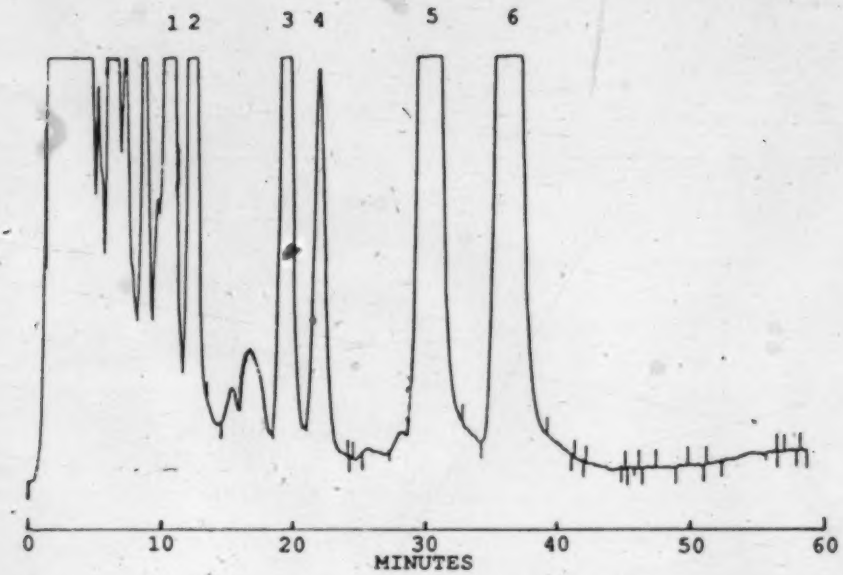


FIGURE 19: High pressure liquid chromatogram of broth from culture of C. echinulata UAMH 4145 spiked with racemic fenoprofen. Derivatization was with L-leucinamide hydrochloride via ethyl chloroformate intermediate. 1 = (R)-keytoprofen, 2 = (S)-ketoprofen, 3 = (R)-fenoprofen, 4 = (S)-fenoprofen, 5 = (R)-4'-hydroxyfenoprofen, 6 = (S)-4'-hydroxyfenoprofen.

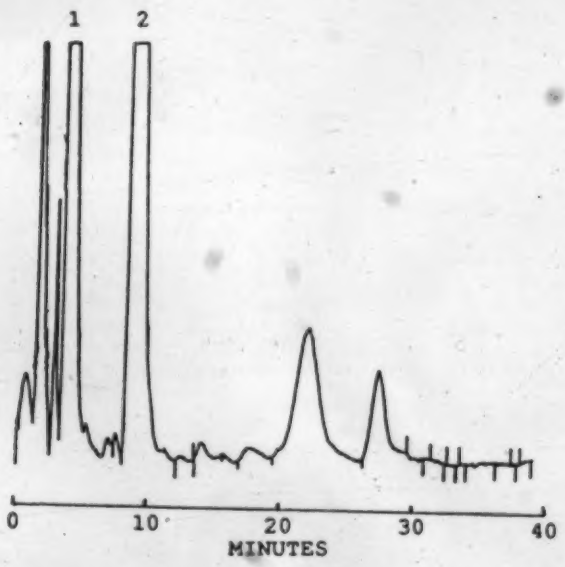


FIGURE 20: High pressure liquid chromatogram of broth from culture of C. echinulata UAMH 4145 spiked with racemic fenoprofen. 1 = 4'-hydroxyfenoprofen, 2 = fenoprofen.

times the area of the earlier peak, and this would correspond to the (S)-enantiomer if the metabolite was to follow the separation characteristics of fenoprofen and ketoprofen (95,120), further paralleling documented human metabolism (95).

In order to determine the actual composition of the metabolites detected in the screen, a large-scale fermentation was carried out in an airlift reactor to produce enough metabolite(s) for MS, ^{1}H-NMR, and IR analysis. In this case, the metabolism of the fenoprofen occurred much more rapidly, apparently due to the increased oxygen delivery by the airlift reactor, thereby enhancing the oxidative biotransformation. Although the fermentation used in the preparative biosynthesis experiment was terminated before the fenoprofen was completely metabolized, another was allowed to proceed to completion whereupon HPLC analysis indicated that of the two metabolite peaks, the later peak was 1.5 times larger than the earlier peak.

A brown oily residue from a single spot separated on preparative TLC which had an Rf of 0.29 appeared to be slightly more polar than fenoprofen which had an Rf of 0.36. This same residue gave rise to the two metabolite peaks seen in the stereospecific HPLC assay. However, upon injection of this residue onto the HPLC using the conditions for non-stereospecific analysis of fenoprofen, only one peak, having a retention time of 3.4 minutes, was detected (Fig. 20). This was consistent with the possibility of it being a

hydroxylated metabolite of fenoprofen which, being more polar, would be expected to elute from a reverse-phase column in less time than fenoprofen, which had a retention time of 8.4 minutes. It also supports the assumption that the metabolites of each enantiomer of fenoprofen were being detected in the stereospecific analysis. This would imply that the metabolism carried out by the organism is itself stereoselective, since the peak area of the later eluting metabolite enantiomer was greater than that of the earlier eluting one.

Analysis of the metabolite by high resolution EIMS (Fig. 21) indicated a compound with a molecular formula of $C_{15}H_{14}O_4$ and a molecular ion of mass 258.0895 which agrees with that calculated for the hydroxylated metabolite of fenoprofen (258.0892). The fragmentation pattern seen also supports this conclusion [m/z (% relative abundance): 258(100), 213(49), 109(23), 91(23), 65(8)] where the ion of m/z 213 arises from the loss of COOH from the molecular ion. Alternatively the loss of $C_9H_9O_2$ from the molecular ion gives rise to $(C_6H_5O_2)^+$ (m/z 109). The ion of m/z 91 is $(C_7H_7)^+$ is formed by rearrangement and in turn loses a molecule of C_2H_2 giving rise to $(C_5H_5)^+$ m/z 65.

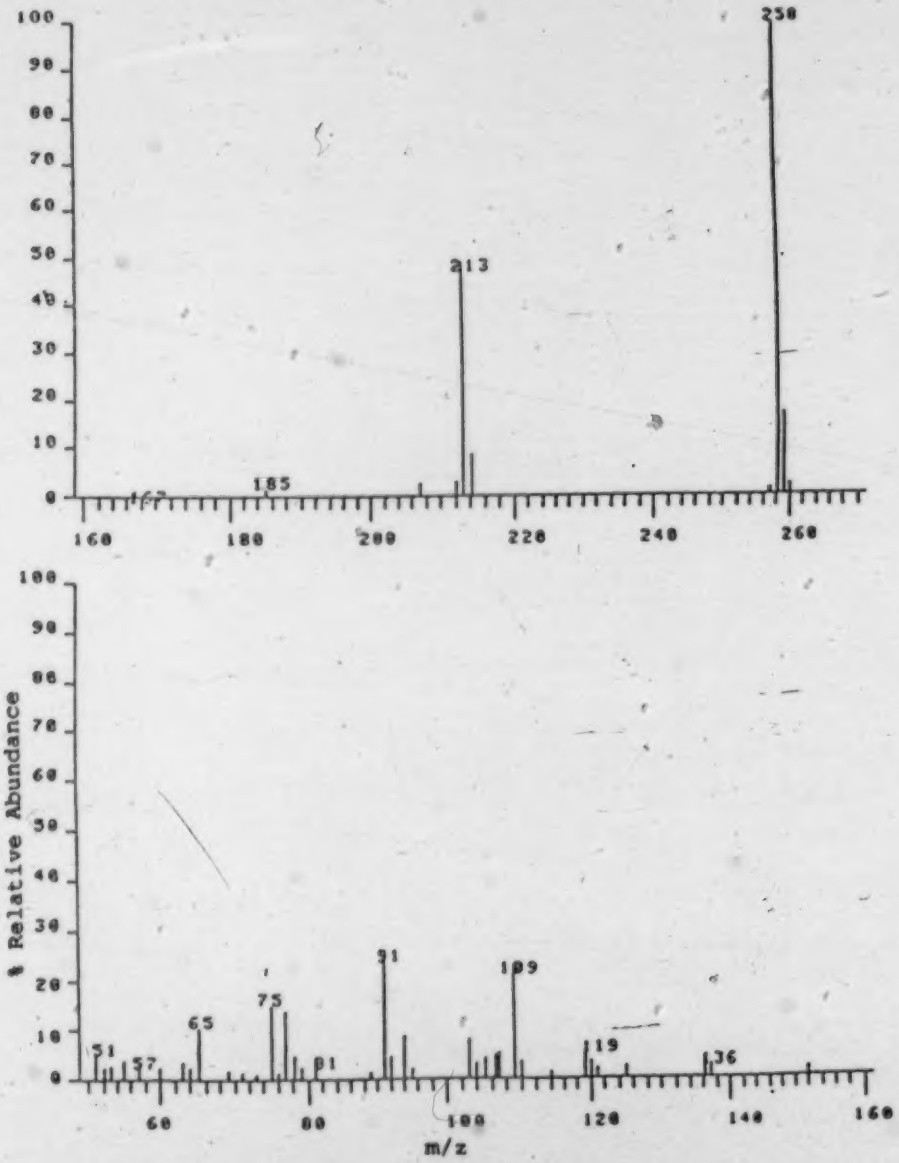


FIGURE 21: High resolution EIMS-line diagram of 4'-hydroxyfenoprofen metabolite from extract of broth from culture of C. echinulata UAMH 4145 spiked with racemic fenoprofen.

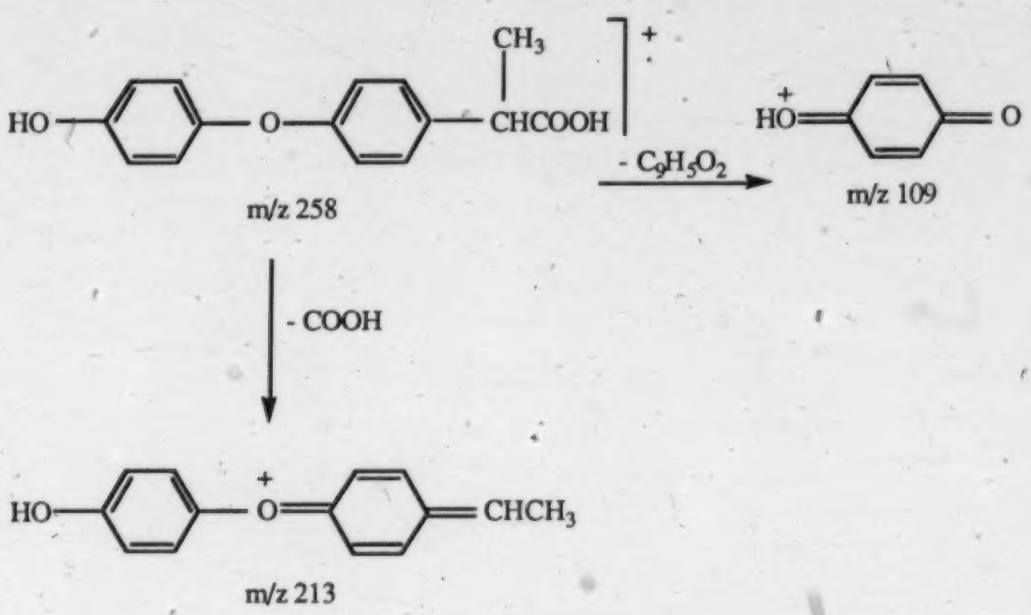


FIGURE 22: Fragmentation pattern of the 4'-hydroxyfenoprofen metabolite from extract of broth from culture of C. echinulata UAMH 4145 spiked with racemic fenoprofen.

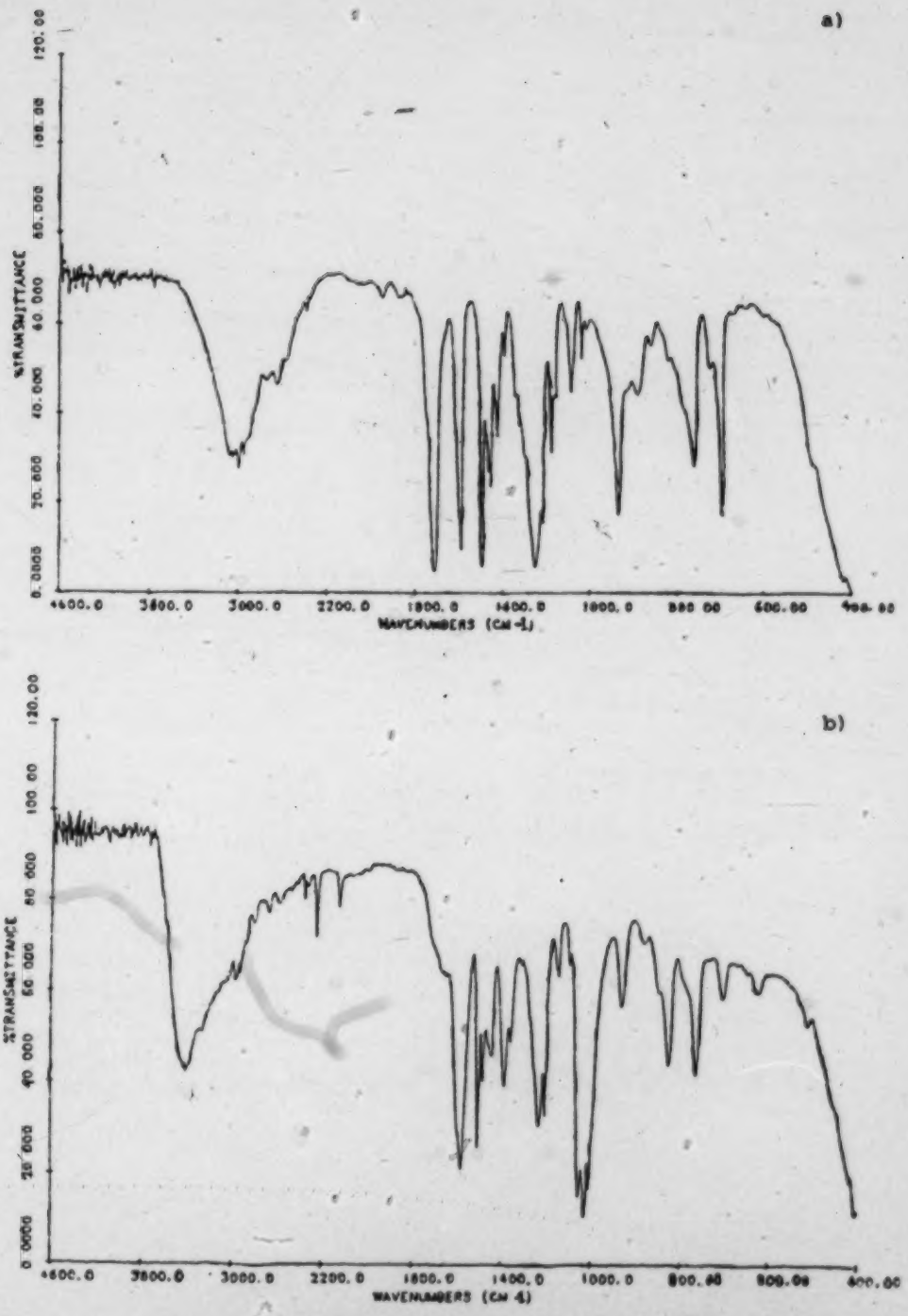


FIGURE 23: Thin-film IR spectra of a) authentic fenoprofen and b) 4'-hydroxyfenoprofen metabolite from extract of broth of C. echinulata UAMH 4145 spiked with racemic fenoprofen.

The IR spectrum of this compound was also consistent with a hydroxylated metabolite of fenoprofen. Having ν_{max} cm^{-1} of 3418, 2639, 2984, 1700 (C=O stretching), 1581, 1400-1500 , and 1240, it differed from authentic fenoprofen in that it had a broad band at 3418 cm^{-1} characteristic of the OH stretching of phenols, and the broad band at 1700 (C=O stretching) was less intense than the sharp band observed in fenoprofen at the same location.

Analysis of the metabolite by 300 MHz ^{1}H-NMR (Fig. 24) gave a spectrum consistent with the *para*-hydroxylated metabolite of fenoprofen. Resonances were assigned as follows: A triplet at 7.18 ppm was consistent with one proton in the 5 position, a doublet at 7.01 ppm with the proton in the 6 position, and a singlet at 6.95 ppm with one proton in the 2 position. The doublet at 6.64 ppm arose from the proton in the 4 position (which occurs further upfield that that of position 6 because of the anisotropic (deshielding) effect on protons *ortho* to the side chain containing a carbonyl group). Four protons in the 2', 3', 5', and 6' positions are nearly equivalent and give rise to four triplets at 6.84 ppm. Finally, a quartet at 3.37 ppm corresponding to the proton attached to the chiral carbon of the side chain and a doublet at 1.27 ppm corresponding to three methyl protons were also observed. A resonance for the OH group is present as a very broad peak of low amplitude at 4.5-5.5 ppm as indicated by the integration seen in Fig. 24.

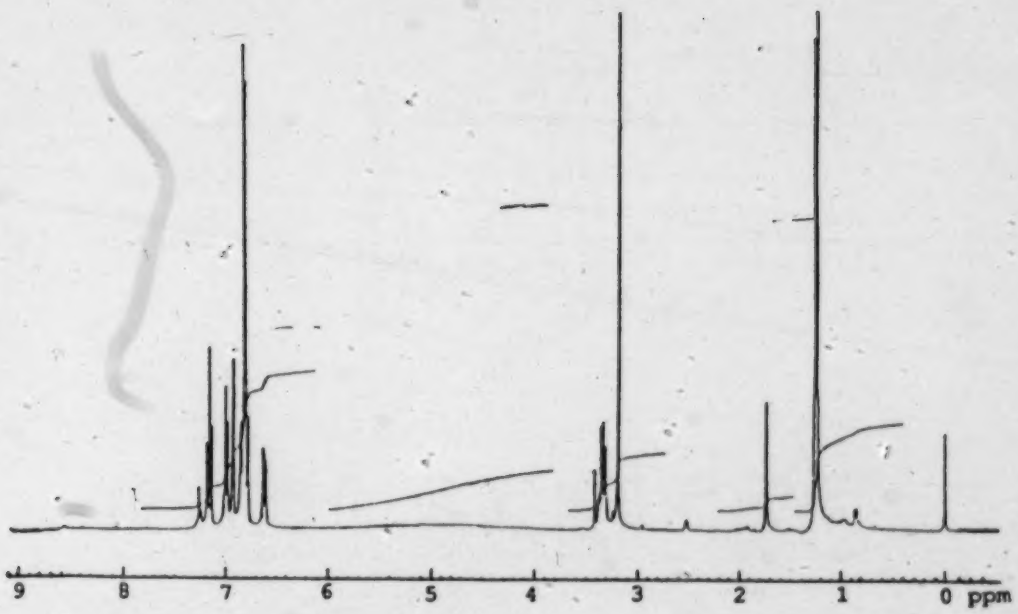


FIGURE 24: 300 MHz ^{1}H-NMR spectrum of 4'-hydroxyfenoprofen metabolite from culture of C. echinulata UAMH 4145 spiked with racemic fenoprofen (in DMSO-d_6).

The proton in the COOH group is not visible because strong deshielding by oxygen would cause it to shift downfield to 10-13 ppm.

It is concluded that C. echinulata UAMH 4145 is able to metabolize fenoprofen to 4'-hydroxyfenoprofen under the conditions investigated. This is an important biotransformation in that it appears to parallel the only phase I transformation reported in the human metabolism of fenoprofen, and it is amenable to scale-up for synthetic purposes. The exclusive synthesis of a *para*-hydroxylated product is not common to all mammals, and since man has been shown to metabolize a variety of compounds in this manner, a microbial model known to carry out this particular biotransformation, and which is condusive to scale-up, could be useful in studying the metabolites of this and other drugs.

In addition, the apparent stereoselective metabolism of fenoprofen by the microorganism, although not nearly as pronounced as in man, where the inversion is almost completely to the (S) enantiomer, merits further investigation as a possible model for stereoselective biotransformation or as a tool in the synthesis of pure enantiomers of drugs and their metabolites.

4.3.4.0 Results and discussion on human metabolism of fenoprofen

Analysis by HPLC, of the TLC fractions of urine extract, showed that the fraction which had an Rf of 0.29 gave two peaks with retention times of 30.5 and 36.4 minutes in the stereospecific assay (this corresponded to peaks seen in the fraction containing the microbial metabolite). Incomplete purification was indicated by a large number of interfering peaks also present. Upon repetition of the preparative TLC procedure using a mobile phase of toluene : isopropyl alcohol (85:15), the fraction having an Rf of 0.44 (corresponding to that of the microbial metabolite) was found to produce the same two peaks on HPLC analysis, while the majority of the impurities appeared to have been removed.

Analysis of the human metabolite by high resolution EIMS (Fig. 25) indicated a mixture of two compounds was present (ie the sample had still not been purified to a single component). In the data of the fragmentation obtained [m/z (% relative abundance): 272(27), 258(13), 227(4), 213(16), 136(19), 109(12), 94(45), 91(100), 65(27)], the molecular ion of m/z 272, having an accurate mass of 272.1050 corresponding to a compound of $C_{16}H_{16}O_4$ could not lose 14 mass units to give rise to a fragment ion of m/z 258. This, then, is another molecular ion whose accurate mass of 258.0987 and $C_{15}H_{14}O_4$ corresponds to the expected metabolite 4'-hydroxyfenoprofen (calculated as 258.0982).

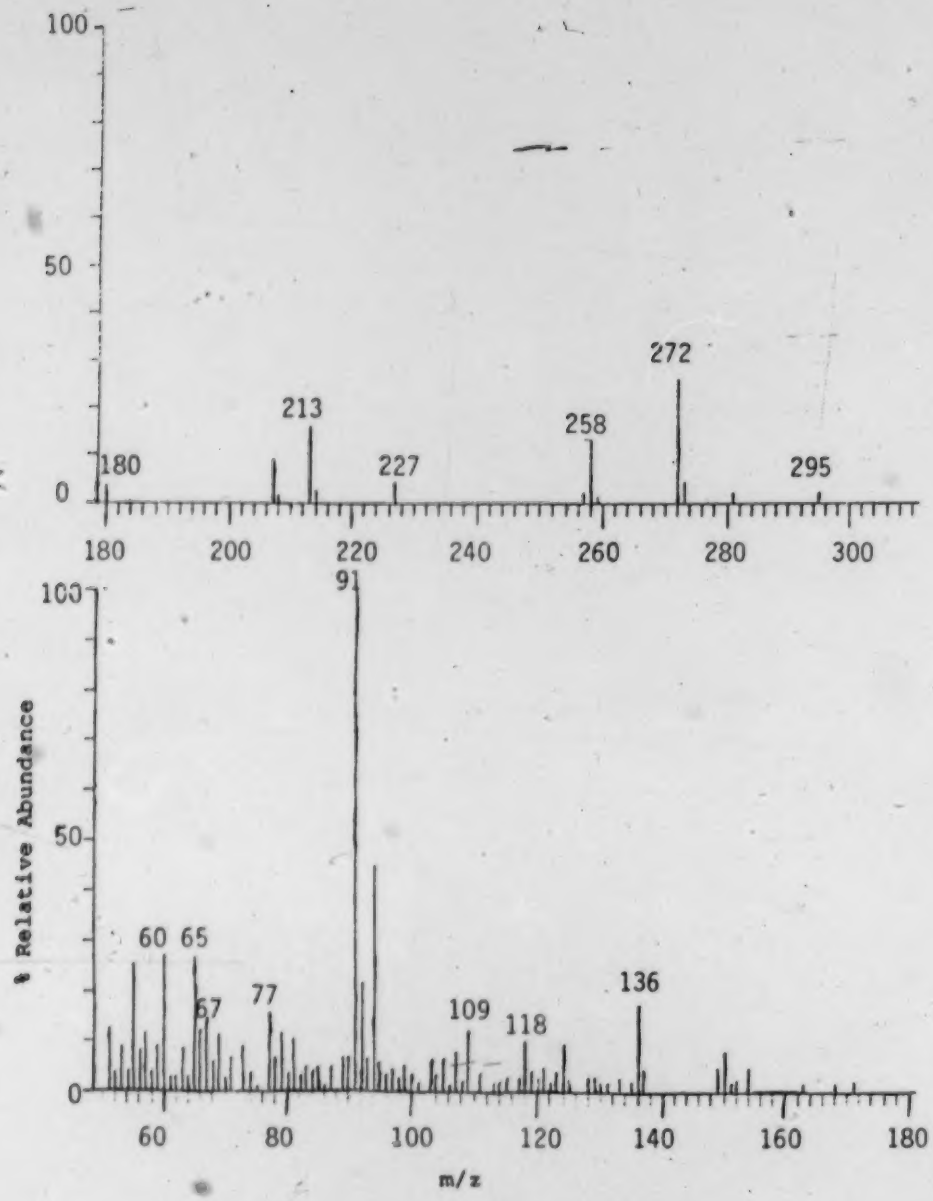


FIGURE 25: High resolution EIMS-line diagram of a mixture of 4'-hydroxyfenoprofen and 4'-methoxyfenoprofen metabolites from extract of human urine collected following the administration of racemic fenoprofen.

Remarkably, the fragmentation pattern still closely resembles that of the purer, microbially produced metabolite. The only exceptions, overlooking differences in relative abundance, are the presence of the second molecular ion of m/z 272 and the ion of m/z 227. This latter ion appears to arise from the loss of COOH by the molecular ion of m/z 272, paralleling a similar loss of COOH by the molecular ion of m/z 258 which gives rise to the ion of m/z 213. It has therefore been proposed that this molecular ion of m/z 272 having the molecular formula $C_{16}H_{16}O_4$ represents the presence of a methoxy metabolite of fenoprofen, which, with a calculated mass of 272.1049, is consistent with the data presented here. To the best of our knowledge, this compound has not been previously reported as a human metabolite.

The IR spectrum of this sample was poor due to its impurity. A large amount of background interference was evident. It did contain broad bands at ν_{max} 3427 cm^{-1} and 1573 cm^{-1} which were similar to those seen in the microbial metabolite, but this evidence was, at best, inconclusive. There was insufficient resolution to identify the presence of an aryl, alkyl ether which would be expected at about 1285-1240 cm^{-1} arising from C-O-C stretching if a methoxy substituent was present.

Analysis of the mixture by ^{1}H-NMR (Fig. 26) produced a spectrum very similar to that obtained for the microbial 4'-hydroxyfenoprofen metabolite. A triplet at 7.14 ppm integrating for a single proton was consistent with one

proton in the 5 position, a doublet at 6.96 ppm with the proton in the 6 position, a singlet at 6.89 ppm with with one proton in the 2 position. A doublet at 6.59 ppm arose from the proton in the 4 position. Four protons in the 2', 3', 5', and 6' positions are nearly equivalent and give rise to four triplets at 6.80 ppm. Finally, a quartet at 3.23 ppm corresponding to the proton attached to the chiral carbon of the side chain and a doublet at 1.19 ppm corresponding to three methyl protons were also observed. This spectrum did differ from that of the microbial metabolite in that it also contained a strong singlet at 3.16 ppm. This would strongly support the presence of the methoxy metabolite which, as indicated by the coupling patterns, must be substituted in the 4' position. One other singlet with a chemical shift of 1.62 ppm was also present.

Based upon the information presented here, we tentatively report the detection of a new 4'-methoxyfenoprofen metabolite, in addition to the 4'-hydroxyfenoprofen metabolite previously described in man. Future endeavors will involve the confirmation of the methoxy metabolite following the successful purification of a sufficient amount of the metabolite for unequivocal spectroscopic analysis.

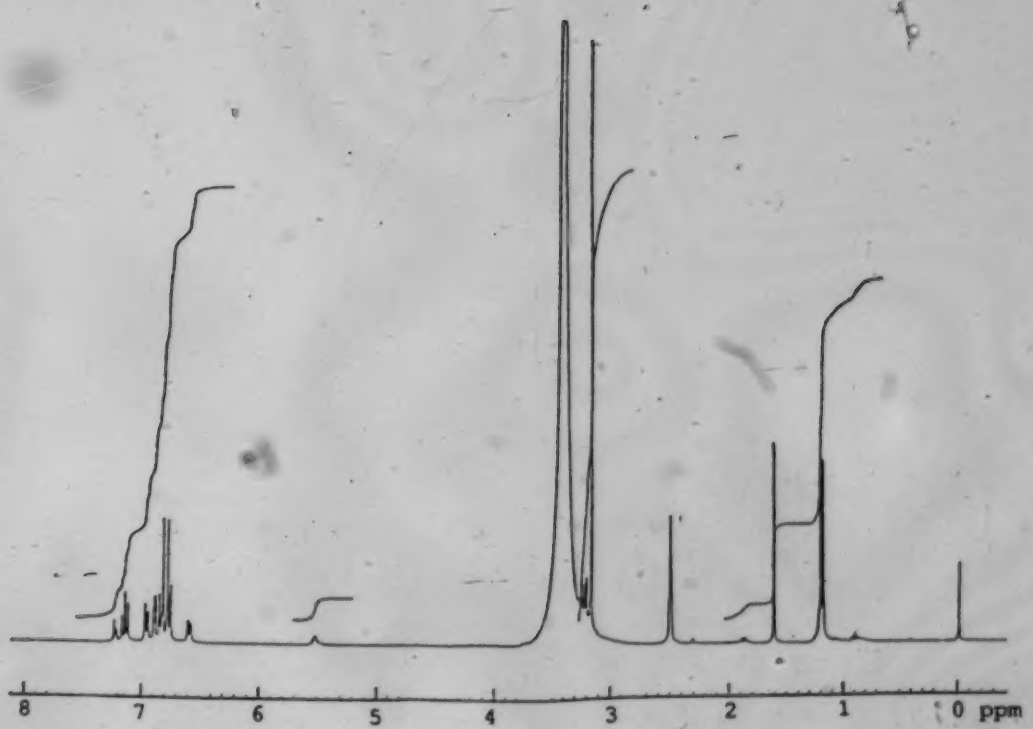


FIGURE 26: 300 MHz ^{1}H-NMR spectrum of a mixture of 4'-hydroxyfenoprofen and 4'-methoxyfenoprofen metabolites from extract of human urine collected following the administration of racemic fenoprofen (in DMSO-d_6).

4.3.5.0 Summary of microbial metabolism of lidocaine, mexiletine, and fenoprofen

Over the course of the metabolism screens the ability of these fungal organisms, Cunninghamella echinulata in particular, to carry out a variety of oxidative biotransformations and their similarity to mammalian phase 1 oxidative biotransformations have been exhibited. These include evidence of the N-dealkylation of lidocaine, the hydroxymethylation and possibly a ring hydroxylation of mexiletine and the *para*-hydroxylation of fenoprofen.

It is curious to note that a specific organism e.g. C. echinulata will metabolize compounds having similarities in chemical structure via different biotransformation pathways in different rates and ratios. For example, racemic N-(n-propyl)-amphetamine (111) was metabolized to give 10 different metabolites. On the other hand, the lidocaine study reported here, and another study on the metabolism of (±)-1-isopropylamino-3-phenoxy-propan-2-ol (113), resulted in the identification of a single, predominant metabolite (dealkylated and hydroxylated respectively). That the metabolism of lidocaine by this microbial system gives a single, predominant, dealkylated product might be explained by the occurrence of substrate inhibition of the organism's saturable hydroxylating system, causing the dealkylation of the substrate to be favored, as occurs in mammalian systems.

In addition, the lack of a similar dealkylation site on mexiletine might also explain why the same organism would carry out the hydroxylation of mexiletine. A simple assay varying the amount of lidocaine added to the fermentation should confirm if concentration-dependant metabolism is actually taking place.

It is quite possible that a significant amount of metabolic variation seen in a particular organism is due to differing fermentation conditions including composition of carbon and nitrogen sources and availability of trace elements. The use of chemically defined minimal media, rather than the more traditional rich soups which are usually based upon meat digests or yeast extract, can aid in the separation, detection and purification of metabolites from the fermentation broth. However, it may also limit an organism's metabolic capacity through a failure to induce as broad a range of cytochrome P_{450} species as possible (e.g., through catabolic repression (11,121)). Oxygen levels in the broth during incubation definitely had an effect on the rate, if not the route, of transformation in the case of fenoprofen metabolism. It would be interesting to see what effect it might have on the metabolism of lidocaine and mexiletine as well. As the conditions for metabolic regulation become more fully documented, and the development of a wider foundation of knowledge in the area of directed biosynthesis is generated, the predictability of any given organism's usefulness as a metabolic model or as a possible source of an

exclusive biotransformation will become an increasingly effective and useful research and synthetic technique.

5.0.0.0 CONCLUSION

Each of the studies reported here exhibits some aspect of the valuable role microorganisms can play in the metabolism of drugs and in the synthesis of drugs and metabolites.

The production of cyclosporine was achieved through the set up and development of a microbial biosynthetic fermentation process using Tolypocladium inflatum Gams UAMH 2880 as the production organism.

In a study involving a screen of fungal organisms in which was investigated their ability to carry out oxidative biotransformations similar to know mammalian phase 1 oxidative biotransformations, C. echinulata UAMH 4145, B. nigricans, and A. flavus UAMH 2679 were observed to carry out such transformations as the N-dealkylation of lidocaine, hydroxymethylation, ring hydroxylation, and N-acetylation of mexiletine, and hydroxylation of fenoprofen. With the exception of N-acetylation of mexiletine, each of these biotransformations have been documented in mammals, which adds to the growing body of data which indicates a similarity between these microbial and mammalian hepatic cytochrome P_{450} systems. C. echinulata appeared to experience inhibition of its ability to carry out aromatic hydroxylation in the lidocaine study. A similar event has been shown to occur in mammalian systems as well, where the hydroxylating enzymes

exhibit saturation kinetics and substrate inhibition in the presence of increased levels of lidocaine.

Scale-up to 1.25 L fermentations was carried out in order to produce enough material to allow confirmation of the *para*-hydroxylation of fenoprofen by C. echinulata through interpretation of IR, 1H-NMR, and high resolution EIMS data from the purified metabolite. In addition, the stereoselective metabolism of racemic fenoprofen was found to occur in C. echinulata, possibly involving inversion from the (R) to the (S) enantiomer, the predominant enantiomer of the metabolite being 1.5 times as abundant as the one seen to elute earlier. This has also been reported in man where almost complete inversion from (R) to (S) is known to occur.

These studies highlight the similarities of oxidative biotransformation capabilities found in microorganisms and mammals which form the basis for using microorganisms as models for mammalian metabolism in order that they might help to predict the formation of possible active or toxic metabolites. Their versatility in scale-up from screens to preparative synthesis of metabolites, as well as their stereoselective and regioselective character, make them a viable alternative to purely chemical synthesis.

The limits of these microorganisms as mammalian models arise from the fact that a single unicellular organism, having no organ systems, cannot faithfully represent the combinations of metabolic transformations likely to be seen in mammalian systems. This limits their role, as a mammalian

model, to screens and preliminary metabolic studies. However, the wide variety of microorganisms, taken in combination, appears to be able to cover the spectrum of mammalian biotransformations. The study of a microbial model may even prove to be an advantage in the study of a specific transformation(s) within a simpler, relatively isolated system in which there is a reduction in the variables affecting the metabolic system, and a greater control of the variables that remain.

The detection of 4'-methoxyfenoprofen as a human metabolite, apparently for the first time, is a discovery the significance of which awaits confirmation of this observation and further study into the abundance and possible activity of this new metabolite.

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